EXPERIMENTAL CELL RESEARCH

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VOLUME 15 · OCTOBER 1958 · NUMBER 2

ACADEMIC PRESS INC., PUBLISHERS
NEW YORK and LONDON

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A METHOD FOR RAPID FRACTIONATION OF PARTICULATE SYSTEMS BY GRADIENT DIFFERENTIAL CENTRIFUGATION

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Received December 10, 1957

In initial experiments on the fractionation of mitochondria into subparticulate constituents, it became apparent that for isolation of the particles, a method more satisfactory than the customary methods of differential centrifugation was needed. The subject of this report is a simple and rapid method for obtaining relatively uncontaminated preparations of particulate cell components by gradient differential centrifugation, in quantities sufficient for further subfractionation. The method depends on the use of a centrifuge distribution head that allows rapid construction of liquid density gradients. Although we have been primarily concerned with the isolation of mitochondria free of nuclei and small particles, the method is applicable, with suitable modification, to a variety of particulate systems.

The resolution of suspensions of particles, such as tissue breis, into fractions of relatively uniform size and density is greatly improved if the centrifugation is accomplished in continuous density gradients [1, 2, 4, 10]. This is because centrifugation anomalies that lead to mixing and cross contamination are largely eliminated [1, 2]. The literature describing the experiences of several workers in adapting gradient differential centrifugation to various systems is reviewed by Anderson [2] and Svensson *et al.* [19]. Papers not mentioned by these authors are by Weber [22], who describes the separation of the particulate components of *Xenopus* eggs in density gradients, and by Thomson and Klipfel [20], who relate enzymatic activity to the size of particles isolated by gradient differential centrifugation from liver breis. Cardinal difficulties with previous methods of gradient differential centrifugation have been (*a*) the time required to produce the gradients and (*b*) the small volume of brei fractionated per tube. In the method previously described by Anderson [2], for example, 2–3 ml of material could be fractionated per tube, and the filling of each required 90 minutes.

[1] Operated by Union Carbide Nuclear Comapny for the U.S. Atomic Energy Commission.

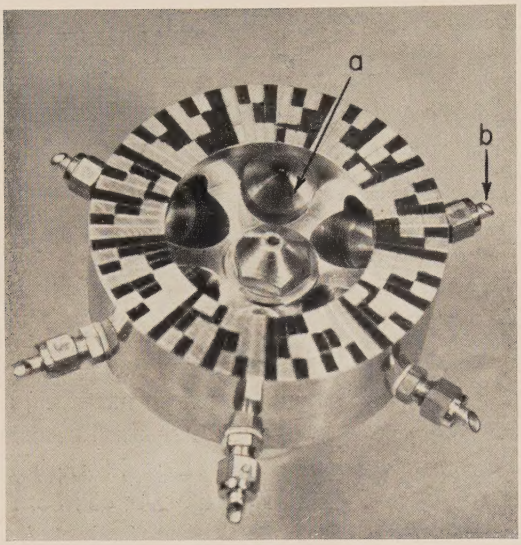


Fig. 1 *A*.

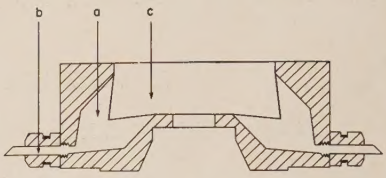


Fig. 1 *B*.

Fig. 1*A*.—Centrifuge distribution head. *a*, Radially oriented concavity; *b*, outlet tube. Scored and painted for stroboscopic speed control.

Fig. 1*B*.—Centrifuge distribution head. *a*, Radially oriented concavity; *b*, outlet tube; *c*, receiving bowl.

Increase in capacity can be achieved only through an increase in the number and in the cross-sectional area of the tubes. However, tubes cannot be filled rapidly with a continuous density gradient without swirling and mixing unless the gradient is stabilized, as in a centrifugal field. We, therefore, explored methods for filling centrifuge tubes during centrifugation and designed a gradient distribution head that apportions one continuously produced density gradient among six 250 ml centrifuge tubes. At 1400 rpm, the distribution head divides the density gradient stream into 140 segments per second, thus ensuring even distribution among the tubes.

MATERIALS AND METHODS

Construction and operation of the distribution head.—The circular distribution head (Fig. 1) is 5 inches in diameter and 2 inches high and was fabricated from type 347 stainless steel. In operation, it is mounted on the shaft of the International Equipment Company's model PR-2 centrifuge resting on the conventional yoke-type centrifuge head No. 259. The distribution head is divided into six equally spaced, radially oriented concavities shown in Fig. 1*A* that are tapered at the bottom and connect with outlet tubes (Fig. 1*B*) projecting from the periphery of the head.

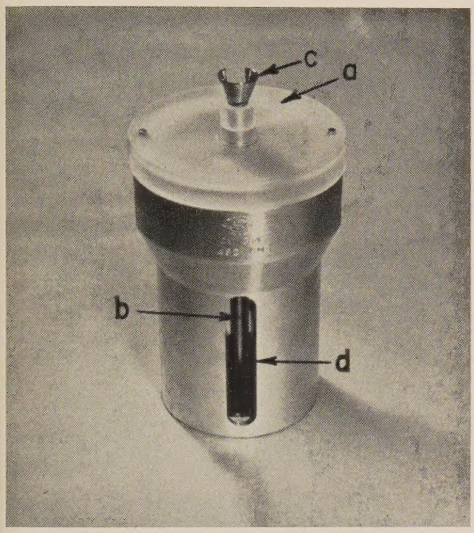


Fig. 2.

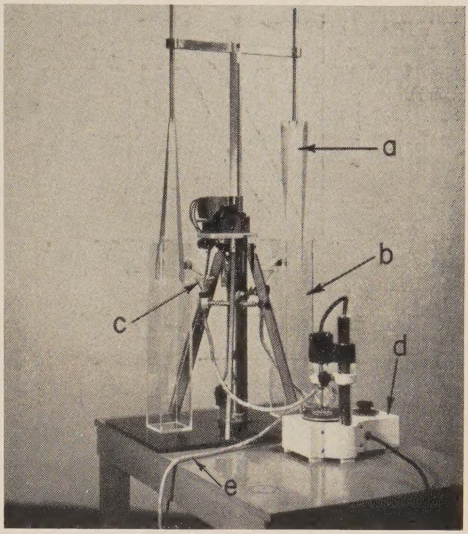


Fig. 3.

Fig. 2.—Duralumin cup. *a*, Lucite cap; *b*, conduit tube; *c*, slotted funnel; *d*, observation slit.
Fig. 3.—Gradient engine. *a*, Lucite blocks; *b*, lucite vessels; *c*, funnels; *d*, high-speed mixer; *e*, conduit to distribution head.

The six duralumin cups that accompany head No. 259 are equipped with lucite caps held in place by brass pins (Fig. 2). The latter support stainless steel tubes (Fig. 2) flared and slotted at the centripetal end. The flared ends of the tubes are designed to enclose the centrifugal ends of the outlet tubes on the distribution head. The centrifugal ends of the conduit tubes extend almost to the bottom of 250 ml Pyrex bottles that are placed inside the duralumin cups (the necks of the bottles have been cut off to permit the use of the lucite caps).

During acceleration, the cups swing to the horizontal position, and the flared ends of the conduit tubes enclose the tips of the outlet tubes. Liquid is introduced into the bowl of the distribution head through a funnel and pipe that extend through the hinged top of the centrifuge. Liquid entering the rotating bowl is distributed equally and continuously into the concavities and is forced outward into the glass bottles.

In operation, the centrifuge is accelerated to 1400–2200 rpm; and the tissue brei, or other suspension, is introduced. This is followed at once by the gradient, beginning with the least dense liquid and progressing to the denser mixture. The brei is thus displaced centripetally, remaining on the surface of the gradient. Mixing is prevented by the centrifugal force. In this manner,

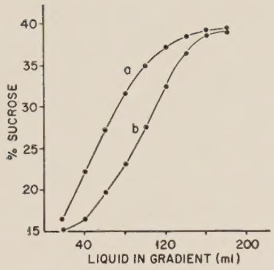


Fig. 4.

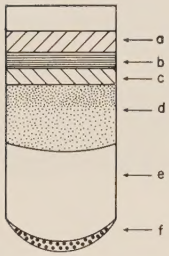


Fig. 6.

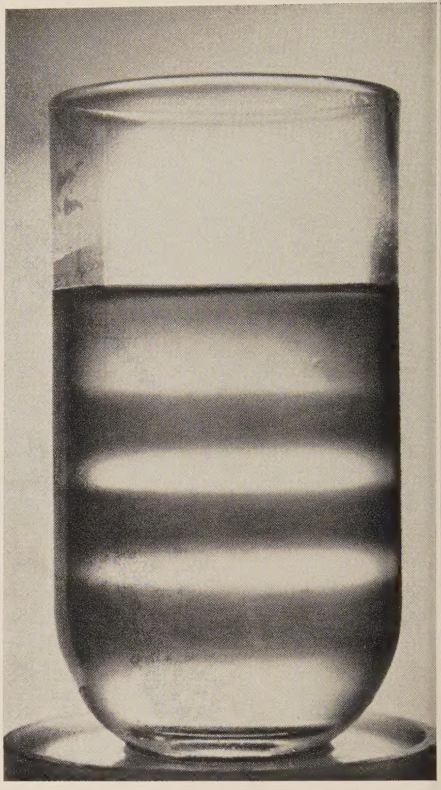


Fig. 5.

Fig. 4.—Curves describing gradient produced by (*a*) pyramid, inverted-wedge system, (*b*) double pyramid system.

Fig. 5.—Stratification of sucrose solutions of eight different densities; alternate solutions were colored with toluidine blue dye.

Fig. 6.—Stratification of rat liver brei in a sucrose gradient. Average particle diameter increases from near the limit of microscopic resolution (*a*) to 1.8 μ (*d*); nuclei found only in (*f*).

the time required for the construction of the sucrose gradient in all six 200-ml glass bottles is less than 10 minutes. For evaluation of the performance of the apparatus, the centrifuge was modified to allow the production of the gradient and the subsequent separation to be observed directly during centrifugation. We did this by cutting slits in the duralumin cups (Fig. 2) and providing a glass port in the bottom of the guard bowl and a hinged top of 1-inch lucite on an International Model PR-2 centrifuge. Illumination was provided by a high-intensity stroboscopic lamp[1] mounted below the guard bowl port. For accurate stroboscopic determination of speed of revolution, the top of the distribution head was scored and painted (Fig. 1*A*) as previously described [1].

[1] Strobolux, Type 648-A and Strobotac, Type 631-B, manufactured by General Radio Company.

Construction and operation of the gradient engine.—The gradients were prepared with a gradient engine developed by Brown [7] (Fig. 3), slightly modified to deliver a gradient of the desired character. The device lowers two lucite blocks of suitable shape (Fig. 3) into two vessels containing liquids of different density. The liquid displaced by the lucite blocks is caught in two funnels and conducted to a high-speed mixer (Fig. 3) to which a flask with three side arms was adapted. It is essential that the two liquids be rapidly and thoroughly mixed. From the mixer, the gradient liquid is conveyed to the bowl of the distribution head.

The shapes of the lucite blocks depend on the nature of the gradient desired. Gradients of two different types were tested for the fractionation of rat liver breis. Fig. 4 (curve 6) shows the sigmoidal curve that characterizes the gradient produced when a lucite pyramid (base: 5.71 × 5.71 cm, height 43.8 cm), suspended upright, descends into 15 per cent sucrose while an identical pyramid, suspended in the inverted position, descends into 40 per cent sucrose. Curve *a* in Fig. 4 was obtained by replacement of the inverted pyramid, which descends into 40 per cent sucrose, with a lucite wedge having the same over-all dimensions.

The composition of the gradient at any instant is given by the ratios of the horizontal cross sections of the lucite shapes at the level of the surface of the liquid. If the upright pyramid and inverted wedge have two sides parallel, the ratios of the two cross sections may be calculated as follows: both shapes have square bases of equal size (b^2) and have the same total height L. The cross section (S^2) at height l for the pyramid is

$$S_p^2 = \frac{b^2 (L-l)^2}{L^2}, \tag{1}$$

and for the inverted wedge

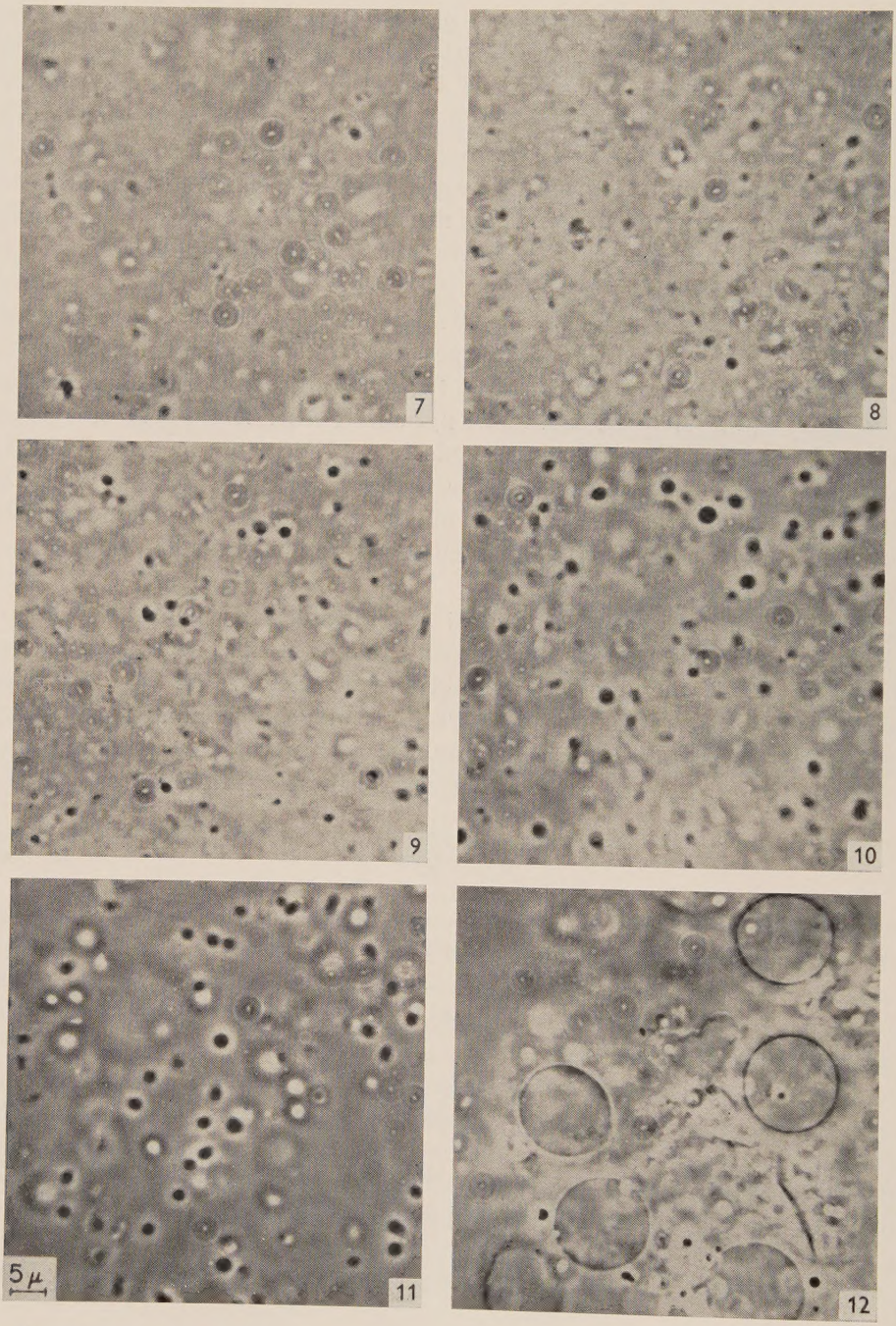
$$S_w^2 = \frac{b^2 l}{L}. \tag{2}$$

Combined, the ratio is found to be

$$\frac{S_p^2}{S_w^2} = \frac{(L-l)^2}{Ll}. \tag{3}$$

The shape of the gradient is easily calculated from expression (3) and the formula for the volume (V) delivered as a function of l:

$$V = \frac{b^2 L}{3} - \frac{b^2 (L-l)^3}{3L^2} + \frac{b^2 l^2}{2L}. \tag{4}$$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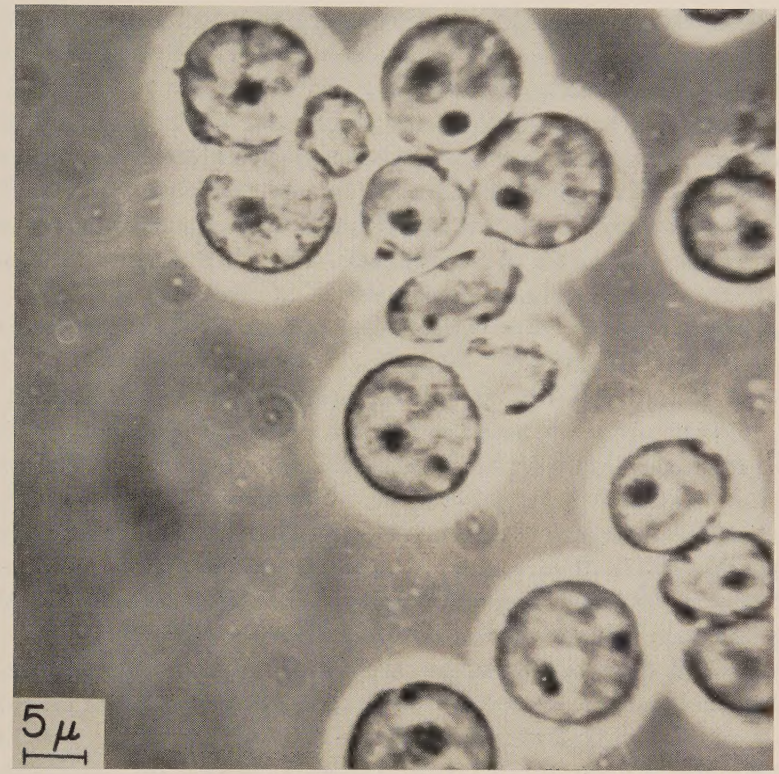

Fig. 13.—Phase photomicrograph of nuclei obtained after recentrifugation of layer *f* in sucrose gradient at 450 × *g* for 10 minutes. Note absence of contamination. Oil immersion, dark-M objective.

Fig. 7.—Phase photomicrograph of layer *a* of stratified rat liver brei. Oil immersion, dark-M objective.

Fig. 8.—Phase photomicrograph of layer *b* of stratified rat liver brei. Average particle diameter, 1.0 μ. Oil immersion, dark-M objective.

Fig. 9.—Phase photomicrograph of layer *c* of stratified rat liver brei. Average particle diameter 1.4 μ. Oil immersion, dark-M objective.

Fig. 10.—Phase photomicrograph of layer *d* of stratified rat liver brei. Average particle diameter 1.8 μ. Oil immersion, dark-M objective.

Fig. 11.—Phase photomicrograph of layer *d* of stratified rat liver brei after centrifugation through gradient a second time. Oil immersion, dark-M objective.

Fig. 12.—Phase photomicrograph of layer *f* of stratified rat liver brei after centrifugation at 800 × *g* for 90 minutes. Note contamination of nuclei by mitochondria. Oil immersion, dark-M objective.

Rat liver breis.—Sprague-Dawley rats were stunned, exsanguinated, and the livers rapidly perfused with Lock's solution. The livers were removed, weighed, and homogenized in 0.25 *M* sucrose plus 1×10^{-4} *M* $CaCl_2$. The total brei volume was three times the liver weight. The brei was strained through four layers of cheesecloth. It was then subjected to gradient fractionation either without further dilution (concentrated brei) or after dilution with 0.25 *M* sucrose plus 1×10^{-4} *M* $CaCl_2$ to a final concentration of one part liver in ten parts of brei (*w*/*v*) (dilute brei). The temperature of the brei and of the sucrose gradient did not rise above 6°C at any time.

The sucrose solutions from which the gradients were constructed usually contained 1×10^{-4} *M* $CaCl_2$.

Chemical estimations.—Ribonucleic acid (RNA) was extracted from freshly isolated mitochondria by the Schmidt-Thannhauser-Schneider procedure [15, 21] and was estimated spectrophotometrically by the orcinol reaction for pentose [6]. A standard curve based on a commercial sample of yeast RNA was used as the reference. Protein was estimated both in fresh, unextracted mitochondria and in the residue remaining after extraction. The analysis was based on the biuret reaction as modified by Weichselbaum [23]. Total nitrogen was estimated by the micro-Kjeldahl method [14].

The chemical data are expressed in amount per million mitochondria. Counts of mitochondria were made as described by Shelton *et al.* [17].

RESULTS

To establish that the operation of the distribution head did not lead to appreciable mixing of the liquid traversing it, we made preliminary tests by introducing into the head equal volumes of solutions of 8 different concentrations of sucrose of specific gravity from 1.03 (10 per cent) to 1.17 (40 per cent). Alternate concentrations were colored with a few drops of toluidine blue dye. Fig. 5 shows the stratification resulting in the receiving bottles. Very little mixing was observed.

The success of fractionation of the breis depended in part on the character of the gradient. Although a gradient characterized by a sigmoidal curve (Fig. 4, plot of concentration vs. volume of gradient) afforded slightly greater stratification of the brei, the various strata were noticeably contaminated by particles that, from microscopic examination, should have been localized elsewhere. A gradient described by curve *a* (Fig. 4) allowed the best resolution of a brei into layers of uniform particle size. The stratification achieved in a typical experiment after 90 minutes' centrifugation at $960 \times g$ (center

of bottle) is shown diagramatically in Fig. 6. The dimensions of the particles and the uniformity of size in samples from the various strata are shown in Figs. 7–13. The centrifugal force developed under the conditions described is not sufficient for satisfactory resolution of the very small particles according to their size and density. Phase microscopic examination of the top layer, *a* (Figs. 6, 7), reveals a heterogeneous population of small particles, with a few large particles and many lipid droplets. The size of the majority of the visible particles is near the limit of optical resolution. Layer *b* (Figs. 6, 8) is rich in particles 0.3–1.6 μ in diameter (average diameter, 1.0 μ). The diameter of the majority of the particles in layer *c* ranges from 1.0–2.1 μ (Figs. 6, 9). The average diameter of these particles, 1.4 μ, is greater than that of the particles in layer *b* but less than that in layer *d*. The particles in layer *d* (Figs. 6, 10) appear to be typical large mitochondria. The size of the particles in this layer is quite uniform (1.6–2.6 μ; average diameter, 1.8 μ). We can achieve even greater uniformity of particle size by removing this layer after the first gradient centrifugation, concentrating it by high-speed centrifugation and centrifuging in a gradient a second time (Fig. 11). An additional indication of the uniformity of layer *d* results from centrifugation at high speed (21,000 $\times g$) after recovery from the gradient. The resulting pellet is devoid of a "fluffy" layer that is generally attributed to concomitant sedimentation of large microsomes [see 16]. Layer *e* (Fig. 6) is almost devoid of particulate material. The cell nuclei are concentrated in layer *f* (Fig. 6) at the bottom of the bottle. Microscopic examination (Fig. 12) reveals that they are highly contaminated with aggregates of large mitochondria. However, recentrifugation of layer *f* in a gradient one or two times (less than 12 minutes' centrifugation time is required to sediment the nuclei at 450 $\times g$) permits the preparation of a relatively uncontaminated batch of nuclei (Fig. 13). Two or more times through the gradient seems to result in some shrinkage and granulation of the nuclei (see Figs. 12, 13), although other media might prevent nuclear damage. All the nuclei seem to be sedimented by this procedure; *no* nuclei were discovered in other layers.

It should be understood that the centrifugal fractionation of a particulate system depends on both size and density of the various particles. Thus the separation, according to size, of a suspension into layers in which the particle size is perfectly uniform cannot be achieved if the particles also vary in density and vice versa. This largely accounts for the range in particle size found in the separate layers.

By this method, the time required for the establishment of a total volume of 1200 ml (six bottles, 200 ml per bottle) of gradient of continuously varying

TABLE I. *Some chemical analyses of mitochondria isolated by differential gradient centrifugation.*

Number of determinations	Substance	Amount per million mitochondria ($\mu g \times 10^{-2}$)
1	RNA	1.1
4	Nitrogen	3.8
4	Protein	21.0

density is less than 10 minutes. A volume of 120 ml of brei (20 ml per bottle), corresponding to 1-2 rat livers, is satisfactorily fractionated in about 90 minutes' centrifugation time. The entire operation may be easily performed in 2 hours.

Since our interest is in the procurement of uncontaminated mitochondria, we have adjusted conditions to permit maximum development of layer *d*. The particulate matter in this layer has been partially characterized chemically as shown in Table I.

DISCUSSION

The method described should be applicable in the fractionation of particulate systems in general. Thus, in addition to the separation of liver subcellular fractions, it may be useful in the resolution of mixtures of cells of different types into groups of cells, each of which is relatively uniform with respect to size and density. This should permit more meaningful analyses of changes in tissues under various conditions by permitting the analysis of changes in several cell types. It should also facilitate studies of the interactions of different cell types under various circumstances. For example, Anderson *et al.* [3] used differential gradient centrifugation for the resolution of suspensions of bone marrow cells into several distinct groups.

Gradient differential centrifugation for fractionation of cell particles for biochemical studies has not been widely used. This may be attributed, in part, to the relatively low yield and complex apparatus required. It should be pointed out that the use of discontinuous gradients for achievement of cell fractionation, such as those used by Kuff and Schneider [12], suffers from the same sources of error as does differential centrifugation in which a gradient is not used.

Work to date [8, 9, 11, 13, 18] indicates that a significant portion of cyto-

plasmic protein may be held in common by microsomes, mitochondria, and the soluble phase. Yet the proteins that are restricted to these sites are likely to be the most interesting for further biological experimentation. Although a number of enzymes are known to be largely localized at certain intracellular sites, we believe, temporarily ignoring enzymatic activity, that a fresh approach to the characterization of cytoplasmic proteins may be rewarding. The availability of an analyzable quantity of reasonably homogeneous starting material should make future studies on the protein composition of mitochondria more meaningful.

SUMMARY

A method is designed for the rapid fractionation of large volumes of suspensions of particles varying in size and density by gradient differential centrifugation. A centrifuge distribution head is used that permits the rapid construction of continuous liquid density gradients since the gradients are stabilized by centrifugal force as they are formed. The application of the method to the fractionation of rat liver breis is described.

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TROPISTIC RESPONSES OF ZYGOTES OF THE FUCACEAE TO POLARIZED LIGHT[1]

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Received December 10, 1957

In 1934, Castle [5] reported that a beam of vertically vibrating light[2] needed to be about 10 per cent more intense than an oppositely directed beam of horizontally vibrating light in order to nullify the latter's tendency to effect phototropic bending of an upright *Phycomyces* sporangiophore. This finding of *polarotropism*, an orientation of growth by the polarization of light, was unique; for previous claims [1, 22, 24] were and remain entirely unconvincing and unconfirmed. Castle interpreted the polarotropic response as arising from differential external reflection. This view now seems incorrect [17]; but so viewed the phenomenon revealed nothing and was not pursued.

Recently, I found a striking polarotropic response of *Fucus* zygotes [16]. Zygotes treated with plane polarized white light coming from both above and below tend to germinate horizontally and in the plane of vibration.

I have here tried to study whether or not the polarotropic response of the zygotes is a variant of their response to unpolarized light. For it might not be; the polarized irradiation might orient growth by directly aligning molecules—a hypothesis which invites examination despite the want of evidence for it.

MATERIALS AND METHODS

Obtaining zygotes.—Receptacles, or fertile fronds, of *Fucus furcatus* were collected at Carmel Point, Carmel, California; those of *Pelvetia fastigiata* at Reef Rock, La Jolla. They were treated and stored as described by Whitaker [33] and Jaffe [15], respectively. Three to six days after collection, the receptacles were transferred to a room held at $15 \pm 3/4$°C where all subsequent operations were carried out; these started immediately with a treatment used to stimulate gametangial liberation.

[1] Contribution from the Scripps Institution of Oceanography. The experimental work was done as a Postdoctoral Fellow in Marine Biology at the S.I.O. Instrumentation was carried out under a contract with the Bureau of Ships. Analysis of the data was carried out at the author's current address, Brandeis University, Waltham, Massachusetts, with the support of both Brandeis and the Office of Naval Research (Contract Nonr 1677 (02) NR 164–406).

[2] The direction of vibration here is that of the electric vector.

For *Fucus*, Whitaker's method [33] of simply immersing the stored receptacles in 15°C sea water sometimes failed in these experiments. For this reason, the following further expedient was resorted to: About one hour after immersion the receptacles were placed about two inches below a 20 watt white fluorescent lamp; marked shedding ensued one to three hours later. The treatment for *Pelvetia* has been described [15].

The receptacles of *Fucus* and of *Pelvetia* were discarded about 45 and 15 minutes respectively after shedding began. It was at about this time of discard that 50 per cent of the egg-bearing capsules were estimated to have broken down. This estimated time was taken as the "time of fertilization". Where illumination proved necessary in subsequent operations, only ruby lamps were employed.

The sexual products were allowed to remain in the dark for about 20 minutes after discard so as to complete the dissolution of the öogonial capsules and the fertilization of the eggs. The zygotes were then thoroughly washed with artificial sea water [16] and suspended in the same medium to form a "stock suspension".

Method for vertical illumination.—The central light source was a 500 watt projection lamp powered through a voltage stabilizer. Most of the infrared component was filtered out of the horizontally emanating light by immersing the lamp in the center of a 0.6 molar ferrous sulfate solution held in a glass cylinder; each horizontal beam traversed 37 mm of this filter before emerging. The filter was water-cooled during lamp operation and adequately protected against oxidation by a layer of mineral oil above it, 0.05 molar H_2SO_4 in it and a piece of brass at its bottom.

Horizontal beams from the central light source traversed windows in each of nine boxes; thence they were deflected downward by 45° prisms through appropriate filter trains so as finally to irradiate each of nine egg-bearing culture dishes with two 17 millimeter square patches of plane polarized approximately monochromatic light of known intensity. The filter train in each box consisted of the following components (starting with the one nearest the light source): (i) An over-all Wratten neutral filter (fine adjustment was provided by changing the distance between the light source and the optical box). (ii) An interference filter. The transmission peaks and half band widths used are listed in Table I. (iii) One or more cut-off filters. (iv) Two 17 mm square windows, one being covered by Wratten neutral filters, whose measured transmission was 10 per cent. (v) A Polaroid type H linear polarizing filter.

The intensities impinging on the eggs in each patch of light were measured by replacing the culture dishes with a suitably masked photronic cell whose output was read off a stabilized indicating amplifier. The light intensity measured as reaching a patch via the window *not* directly above it never exceeded 3 per cent of the total reaching it. The photometric setup was calibrated in relative energy terms by comparing its reaction with that of a thermopile (whose output was read off a galvanometer) to light beams of the same spectral composition used in treating the eggs. Tests using appropriate extra filters showed that the light beams used were free of infrared. The absolute energies used were determined by calibration with a Bureau of Standards lamp.

Method for horizontal illumination. The light source for the lower intensities used was a 15 watt, 6 volt tungsten filament lamp. Its image was focused through 9 cm of 0.04 *M* $CuSO_4$ + 0.04 *M* HCl onto a 1 cm square of white paper. This then acted as a secondary source of white light free of infrared and of polarization. For doses of 10^{18} quanta/cm^2 or greater, the source was changed to a 30 watt sealed spot lamp

TABLE I. *Response of* Fucus *zygotes to monochromatic plane polarized light coming from above.*

The dose is expressed as quanta/cm². For all values of $V_2 \geq 44$ %, the per cent of outgrowths classified as up averaged 1.0 ± 0.8; for $12\ \% < V_2 < 44$ %, per cent up $= 3 \pm 2$ %; for $V_2 < 12$ %, per cent up $= 8 \pm 5$ %.

Peak λ (mμ)	Half band width	Egg batch	% bipolar alignment, V_2, for dose of:			% twins for dose of:		
			10^{18}	10^{17}	10^{16}	10^{18}	10^{17}	10^{16}
393	14	a		55±5	44±6		23±4	10±3
		c		61±4	31±6		25±4	16±3
425	9	a		60±4	61±4		30±4	14±3
		c		64±4	49±4		31±4	28±3
436	9	a		71±4	63±4		26±4	22±4
		c		64±4	59±4		22±3	20±3
454	10	c		62±4	57±4		24±4	14±3
459	9	a	67±4	61±4		24±5	46±5	
		c		75±3	60±5		43±4	19±4
483	9	a	80±3	63±3		35±5	36±5	
		c		78±3	61±4		29±4	22±3
499	12	a	77±3	73±3		40±6	35±5	
		c		60±4	42±5		20±3	10±3
522	9	a	65±4	30±7		34±5	8±3	
		b	65±4	37±6		12±3	8±3	
		c	62±4	25±6		25±4	14±3	
546	8	a	12±7	−6±7		4±2	7±2	
		c	21±6			8±2		
567	17	a	33±6	17±8		7±3	6±3	
		b	27±7	8±7		3	3	
579	4	b	−11±7	9±7		0	2	
605	13	b	−1±7			2		
627	16	b	−2±7			0		
650	8	b	−1±7			0		
662	9	b	2±8			0		
690	13	b	−2±7			2		
710	13	b	−19±6	−5±7		3	2	
Dark		a		0±7			4±2	
		b		−9±7			2	
		c		−1±6			14±3	

without a depolarizing paper in its beam. The test eggs lay in horizontally placed rectangular chambers made of microslides. A beam from the source traversed a Polaroid H filter and/or a Wratten neutral filter where appropriate, descended upon one side of each chamber at about $5\frac{1}{2}°$ to the horizontal, and passed through a window so as to illuminate directly only the bottom of the chamber, whence it was totally reflected. Thus each egg was illuminated by two beams whose resultant direction was exactly horizontal. It is estimated that no more than 1 per cent of the "unpolarized" light used in some cases was polarized by reflection before striking the eggs. Light intensities were mainly regulated by changing the distance between the chamber and the source, and were measured using a photronic cell calibrated against a thermopile. On the basis of the emission spectrum of a 2780° K tungsten filament lamp [32] and a measurement of the filter's transmission spectrum, it is estimated that about $\frac{1}{5}$ of the radiant energy striking the eggs lay between 400 and 500 mμ, virtually all the rest lying in the longer wavelength visible.

Analyzing outgrowth directions.—Tenfold magnified shadowgraphs were made of all cultures four days after fertilization. The initial angles of rhizoidal outgrowth as projected in the horizontal plane, or plane of the shadowgraph, were then measured. For the experiments with vertical and equatorial illumination, this analysis was restricted to those embryos whose original centers lay more than 380 mμ or about five egg diameters from their nearest neighbors. In cases where an outgrowth had originated so close to the top of a zygote as to entirely hide its thickened origin, it was classified as "up".

RESULTS

Alignment within the plane perpendicular to the direction of illumination.—As previously reported [16] the polarotropic response was effected by illumination coming from both above and below. A subsequent exploratory experiment showed a similar response to illumination from above only. With this in mind, the effects of treating *Fucus* zygotes before germination with different wavelengths of polarized light coming from above only were investigated. One resultant distribution of outgrowth angles is plotted in Fig. 1 *a*.

To characterize the degree of bipolar alignment of each measured distribution, the parameter, V_2 was calculated [7]:

$$V_2 = \Sigma p \cos 2\theta,$$

where p = the percentage of all outgrowths whose projection in the horizontal plane originated at an angle θ to the vibration axis. (Outgrowths classified as up were omitted in the calulation of V_2.)

Note that for a distribution perfectly aligned in the vibration axis, $V_2 = +100$ per cent; for one perfectly aligned perpendicular to this axis, $V_2 = -100$ per cent; for a uniform distribution, $V_2 = 0$ per cent; for a random distribution, $V_2 = 0 \pm 70 \times 7$

per cent/$\sqrt{N}$, where N is the total number of outgrowths not pointing up [27]. The "vector method", using V_2, extracts much more information from the data than one of simply dividing it into the two categories, $-45°$ to $+45°$ and $+45°$ to $+135°$. Theoretically, it would require the measurement of two to four times as many angles (as V_2 varies from 0 to 80 per cent) to attain the same accuracy with a two category method as with the vector method (see below). The theoretical variances of V_2,

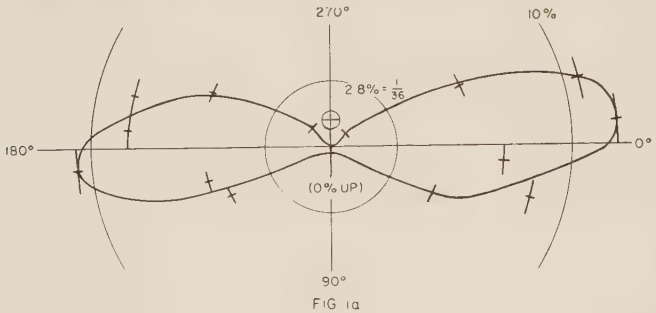

FIG. 1a

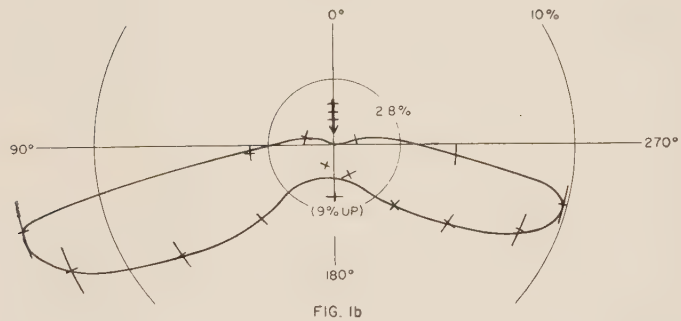

FIG. 1b

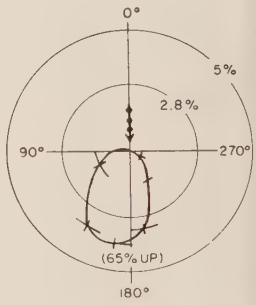

FIG. 1c

Fig. 1.—Illustrative distributions of outgrowth directions of *Fucaceae* eggs in response to treatment with plane polarized light. Per cent of all outgrowths per 10° interval plotted vs. angle. 1*a*—*Fucus* eggs treated with 10^{17} quanta of 483 mμ polarized light coming from above (egg batch c). 0°–180° axis marks plane of vibration. $V_2 = 78$ per cent; $\theta_2 = 0.5°$; $V_1 = 6$ per cent. 1*b*—*Pelvetia* eggs treated with white light horizontally polarized and coming horizontally in the direction of the arrow. 10^{17} quanta of the light lay between 400 and 500 mμ. 1*c*—Same as 1*b* except vertically polarized.

$\sigma(V_2)$, were read off a plot of $\sigma(V_2)$ vs. V_2 constructed for circular normal distributions. These have the form [13]:

$$F(\theta) = \frac{e^{K \cos 2\theta}}{2\pi I_0(K)},$$

where K is a parameter characterizing the degree of alignment.[1] The graph was made using the following two characteristics of circular normal distributions, taken from

[1] $I_0(K)$, $I_1(K)$, and $I_2(K)$ are Bessel functions of the first kind of pure imaginary argument.

Gumbel *et al.* ([13], p. 139) and from Greenwood and Durand ([12], p. 236) respectively:

$$V_2 = I_1(K)/I_0(K)$$

$$\sigma(V_2) = \frac{1}{\sqrt{N}} \cdot \sqrt{\frac{1}{2} + \frac{I_2(K)}{2\,I_0(K)} - \left(\frac{I_1(K)}{I_0(K)}\right)^2}.$$

Evidence that the circular normal distribution is an adequate model is provided in Fig. 2. In order to reveal the shape of the empirical distributions, the largest possible sample was obtained as follows: Those eight distributions for which V_2 happened to lie between 60 and 62 per cent were pooled. Furthermore, each group of four symmetrical ten degree intervals (e.g. 0–10°, 170–80°, 180–90°, and 350–60°) were pooled. In Fig. 2, the histogram represents this empirical data and the smooth curve is taken from that circular normal distribution for which $V_2 = 61$ per cent.

The main result of this investigation appears in Fig. 3*a*, where the bipolar alignment parameters, V_2, are so arranged as to allow the drawing of an action spectrum. The doses employed in all the treatments whose results are listed across from a given dosage were equalized within about 10 per cent. Each dose was applied by means of a ten hour long constant intensity treatment starting two hours after fertilization. (Under comparable conditions, the first zygotes are reported to begin germination 10 to 14 hours after fertilization [34].) In Table I, these same data appear together with estimates of standard deviations and other accessory information.

Consideration of Fig. 3*a* and Table I indicates that a maximum of sensitivity of the eggs toward the growth aligning effect lies between 435 mμ and

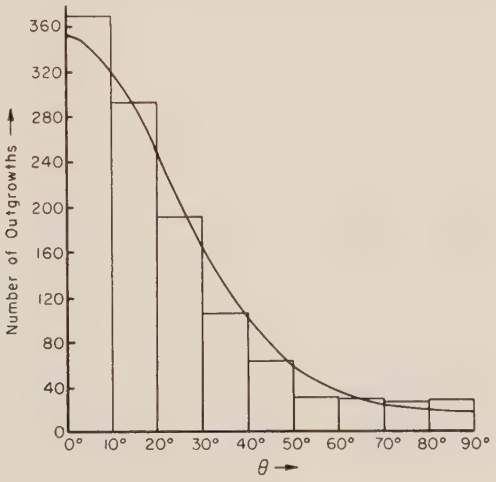

Fig. 2.—Exact shape of distributions of *Fucus* outgrowth directions in response to polarized light coming from above.

490 mμ. In this region the total dose required to produce 60 per cent orientation is 10^{16} quanta/cm² which is 4×10^4 ergs/cm² at 480 mμ. The sensitivity falls relatively slowly toward short wavelengths; at 400 mμ it is about 1/10 maximal. But it falls precipitously toward long wavelengths; at 522 mμ,

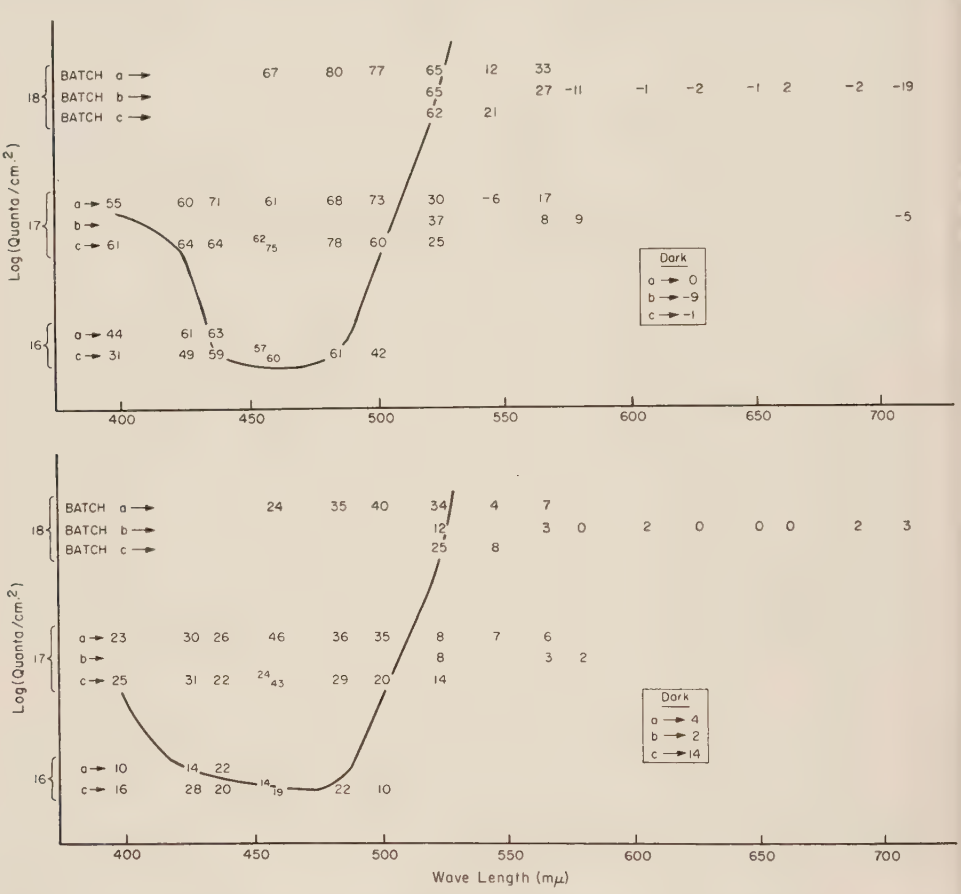

Fig. 3.—Wavelength dependence of response of *Fucus* eggs to plane polarized light coming from above. Fig. 3*a* (above): Alignment response, V_2. The curve is drawn through interpolated doses giving 60 per cent alignment. Fig. 3*b* (below): Twinning response. The curve is drawn through interpolated doses giving 25 per cent twinning.

it is about 1/100 maximal; at 546 and 560 mμ, no more than 1/1000 maximal; between 579 and 710 mμ, no more than 1/10,000 maximal.

It has been assumed that the central tendency of the outgrowths is exactly 0°. Analysis of the present data establishes this point with considerable preci-

TABLE II. *Evidence that outgrowth distributions lack any unipolar component.*

	Strong bipolar orientation ($V_2 = 60 - 80$ %)	Weak bipolar orientation ($V_2 = -20$ to $+20$ %)
Empirical $\sigma(V_1)$	7.0 %	5.9 %
Expected $\sigma(V_1)$	7.7 %[b]	6.9 %[a]
Empirical $\sigma(W_1)$	3.5 %	6.2 %
Expected $\sigma(W_1)$	3.5 %[c]	6.9 %[a]

[a] From Rayleigh distribution [27].
[b] Weighted average between Rayleigh and binomial distributions.
[c] For a mixture of independent Rayleigh and binomial distributions.

sion. The central tendency of each distribution was first characterized by the parameter:

$$\theta_2 = \tfrac{1}{2} \arctan\left(\frac{\Sigma p \sin 2\theta}{\Sigma p \cos 2\theta}\right).$$

The results were then averaged for all 22 distributions which showed at least 60 per cent alignment, yielding the value, $+0.30 \pm 2.8°$.

Moreover, the above data concerns a tendency to grow out either in one direction or its opposite. In view of the symmetry of the known directive influences—light vibration plane, light direction, substratum plane, and residual group effects—it is to be expected that there will be an equal tendency to develop in any two opposite directions; this too is well confirmed by analysis. The requisite information was obtained by first calculating both components of the *unipolar* alignment vector for each distribution:

$$V_1 = \Sigma p \cos\theta, \qquad W_1 = \Sigma p \sin\theta.$$

Table II shows the good agreement between their empirical standard deviations from zero and their theoretical ones as estimated on the basis of random fluctuations.

Alignment within the plane containing the light direction.—Zygotes of *P. fastigiata* were illuminated with white light from one horizontal direction during the period from two to nine hours after fertilization. (Preliminary experiments showed that some zygotes first begin to germinate about 10 to 12 hours after fertilization under similar conditions.)

TABLE III. *Alignment of germination of* P. fastigiata *zygotes by unilateral unpolarized light.*

The dose given is of the estimated fraction of light lying below 500 mμ. 180° indicates germination directly away from the light; 0°, toward it. Outgrowths classified as "up" were not included in the totals. Temperature is 15°C except as noted.

	Dose (quanta/cm²)					
	0	10^{14}	10^{15}	10^{16}	10^{17}	10^{19}[b]
Light on (hrs. after fertiliz.)	[a]	2	2	2	2	1½
Light off (hrs. after fertiliz.)		9	9	9	9	3½
% of outgrowths: 0–90°	47±4	49±5	43±6	19±3	6±2	19±2
90–135°	31±4	29±5	39±6	54±4	43±4	28±3
135–180°	22±4	22±4	19±4	27±4	51±4	53±3
160–180°	11±3	6±2	7±3	10±3	20±3	

[a] Dark control.
[b] $T = 16°C$.

In this case, it is first necessary to consider the response to *un*polarized light. Consider Table III. Compare the dark control with the response to 10^{16} quanta/cm². It will be seen that the response to this intensity is *not* an aggregation of outgrowths in the vicinity of the rear pole, or 180°; rather, it is a tendency to accumulate outgrowths in the *subequatorial* zone between 90° and 135°. At 10^{15} quanta/cm², a weaker, but still significant subequatorial response is seen. However, at 10^{17} and 10^{19} quanta/cm², a rear pole response appears.

In Table IV, find the comparable responses of *F. furcatus* zygotes as reported by Whitaker and Lowrance [34]. 10^{16} quanta/cm² of unilateral unpolarized illumination yields a clear subequatorial response when applied between 9 and 16 hours after fertilization; however, earlier or more intense treatments yield a rear pole response.

The responses to horizontally directed polarized light may now be considered. Fig. 1*b* shows an illustrative outgrowth distribution—that in response to a dose of *horizontally* vibrating light of which 10^{17} quanta/cm² lay between 400 and 500 mμ. Evidently these outgrowths are strongly concentrated around a subequatorial angle. Fig. 1*c* shows another illustrative outgrowth distribution—that in response to the same dose of *vertically* vibrating light. Sixty-five per cent of these zygotes grew "up", the rest tending to

TABLE IV. *As Table III, for* F. furcatus.

(Data of Whitaker and Lowrance [34].)

Dose ...	10^{16}						10^{16}						10^{17}
Light on	[a]	5	6	7	8	9	10	11	12	13	14	15	2
Light off	[a]	6	7	8	9	10	11	12	13	14	15	16	27
0–90°	49±3	38	15	2	5	8	8	10	15	14	23	31	0
90–135°	25±2	28	36	46	52	68	72	66	62	66	62	52	8
135–180°	26±2	34	49	52	43	24	20	24	23	20	15	17	92

[a] Averages of 14 observations of zygotes treated for one hour with 10^{16} quanta/cm² during the *insensitive* periods from 2–15 and 16–27 hours after fertilization. Their tabulated empirical variances appear to be good gages of those of the other figures.

develop directly away from the light. (A zygote cannot grow downward because of the hard substratum.)

Between 10^{15} quanta/cm², the lowest dose eliciting a non-random response, and 5×10^{19} quanta/cm², the highest dose tested, the responses did not differ qualitatively from the above illustrations. Hence, for the purpose of rapidly visualizing the response to plane polarized light over this range, Fig. 1*a*, 1*b*, and 1*c* may be considered as three orthogonal views of the same distribution. In brief, this distribution is shaped like a wing nut; the wings lie in the vibration plane and point at two symmetric subequatorial angles.

Now, to more precisely describe the responses to horizontally directed lights, both polarized and unpolarized, the distributions were characterized by two bipolar parameters: ϕ_2, a measure of central tendency, and L_2, a measure of the degree of orientation around ϕ_2.

$$\phi_2 = \tfrac{1}{2} \arctan \left[\frac{\Sigma [p(\phi) + p(-\phi] \sin 2\phi}{\Sigma p(\phi) \cos 2\phi} \right],$$

$$L_2 = \sqrt{\Sigma [p(\phi) + p(-\phi)] \sin 2\phi]^2 + [\Sigma p(\phi) \cos 2\phi]^2}.$$

where $p(\phi)$ is the percentage of all outgrowths whose projection in the horizontal plane originated at an angle ϕ to the direction of illumination.

ϕ_2 and L_2 are parameters descriptive of behavior in the plane of the light direction corresponding to θ_2 and V_2 in the plane perpendicular to the light direction. For the distribution shown in Fig. 1*b*, $\phi_2 = 115°$ and $L_2 = 70$ per cent. These parameters furnish a somewhat misleading description of the

TABLE V. *Response of* Pelvetia *zygotes to unilateral white light.*

In the experiment using egg batch *A*, 140 to 290 eggs were counted per treatment; for batch *B*, 80 to 150. (Distributions characterized by ϕ values in parentheses are not significantly different from random ones.)

	Quanta/cm² (400–500 mμ)							
	0	10^{14}	10^{15}	10^{16}	10^{17}	10^{18}	5×10^{19}	0
Egg batch ...	*A*	*A*	*A*	*A*	*A*	*B*	*B*	*B*
Polarization:								
Horizontal								
ϕ_2	(102°)	(164°)	106°	111°	115°	117°	128°	(51°)
L_2	6	7	17	52	70	62	76	3
% Up	38	44	44	19	9	20	5	62
None								
ϕ_2	(102°)	95°	104°	122°	137°			
L_2	6	10	21	26	42			
% Up	38	44	45	43	34			
Vertical								
ϕ_2	(102°)	(78°)	125°	137°	152°	145°	169°	(51°)
L_2	6	3	10	16	21	9	31	3
% Up	38	44	47	60	65	85	65	62
ϕ_1	(150°)	(27°)	160°	186°	168°	185°	179°	(245°)
L_1^*	4	4	32	39	71	71	91	9

responses to vertically polarized light since these are unipolar. Hence these latter were likewise characterized by unipolar parameters:

$$\phi_1 = \arctan\left[\frac{\Sigma p \sin\phi}{\Sigma p \cos\phi}\right],$$

$$L_1^* = \frac{1}{1-p(u)}\sqrt{(\Sigma p \sin\phi)^2 + (\Sigma p \cos\phi)^2}.$$

Note that ϕ_1 and L_1^* are measures of the behavior only of those outgrowths not pointing "up". For the distribution shown in Fig. 1*c*, $\phi_2 = 152°$, $\phi_1 = 168°$, $L_2 = 21$ per cent, and $L_1^* = 71$ per cent.

Now consider Table V in which these characterizations of the responses to horizontally directed light are presented.

Two main points can be extracted from it: (i) The polarized light response and the subequatorial response to *un*polarized light fade out over about the

same intensity range. Fig. 4 shows this economically. In it there is plotted against intensity, a clear measure of the response to the *polarized* character of the light (the difference between L_2 for horizontally and for vertically vibrating light), and a comparable measure of the response to unpolarized light (the difference between L_2 for unpolarized light and the dark control). (ii) The shift with increasing dose of unpolarized light from a subequatorial to a rear pole response is paralleled by a slow drift toward higher angles of the polarotropic response: ϕ_2 increases 22° as the dose of horizontally vibrating light increases 5×10^4 times.

Alignment after equatorial illumination.—*Fucus* zygotes were treated from all directions in a plane with equal doses of unpolarized white light, one third of which lay below 500 mμ. They were exposed at 11°C from 2 to 24 hours after fertilization. In essence the technique was to support the zygotes at the center of a doughnut-shaped fluorescent lamp, with appropriate depolarizing neutral filters and hoop-shaped slits interposed between lamp and zygotes.

The zygotes so treated tended strongly to germinate perpendicular to the plane of illumination. Thus in two cases in which the total dose was about 10^{17} quanta/cm^2, V_2 was equal to 65 ± 9 per cent and 78 ± 7 per cent.

Twinning.—Many *Fucus* embryos developed an outgrowth originating from each of two separate, and usually opposite, regions of the zygote. Such embryos were classified as twins. The percentage of twins as a function of the wavelength and intensity of the polarized light used is presented in Fig. 3*b* and Table I. The action spectrum for this response is readily consistent with that for the alignment effect, but the data are more variable so that the wavelength of maximum effectiveness can be restricted only to the broader region between 410 and 500 mμ.

In this polarotropism study, the *Pelvetia* zygotes never developed two normal outgrowths, though rare embryos showed both a normal and a minute, abortive one. For some entirely obscure reason, in other experiments executed four years before, up to 15 per cent of cultures of *Pelvetia* zygotes sometimes developed into twins.

DISCUSSION

The hypothesis that the polarized irradiation orients growth by directly aligning molecules must be rejected. For two results support what is in any event a simpler assumption—that the orientation of the zygotes' growth by unpolarized and by polarized light are both variants of the general phenomenon of phototropism. First, the zygotes' polarotropic action spectrum (Fig. 3*a*) fits what is known of their tropistic response to unpolarized light

TABLE VI. *Comparison of wavelength dependence (in the visible) of polarotropism with phototropism.*

Phenomenon	Material	Most effective wavelengths	Sensitivity: yellow/peak	Authority
Phototropism	*Phycomyces* sporangiophore	400–460	$\leq 10^{-3}$	Castle, 1931 [4]
,,	*Coprinus* sporophore	400–465	$\leq 10^{-3}$	Boriss, 1934 [2]
,, (low threshold response)	*Avena* coleoptile	435–450, (465–480)[a]	$\leq 10^{-4}$[b]	Johnston, 1934 [18] Curry, 1957 [8] Shropshire, 1957 [29]
Phototropism	*Pilobolus* sporangiophore	430–490	$\leq 10^{-1}$	Bünning, 1937 [3]
Polarotropism	*Fucus* zygote	435–490	$\leq 10^{-4}$ to 10^{-3}	This paper

[a] Secondary peak.
[b] [31].

(it cuts off above 500 mμ for *Cystoseira* [25]), and to all other phototropic spectra (Table VI). Secondly, the polarotropic response and the subequatorial response to unpolarized light fade out over about the same intensity range (Fig. 4).

From a developmental viewpoint, the most revealing residuum is that the polarized light treatment can induce up to half the zygotes to grow into twins. For this implies that the polarity is not established through the rotation of

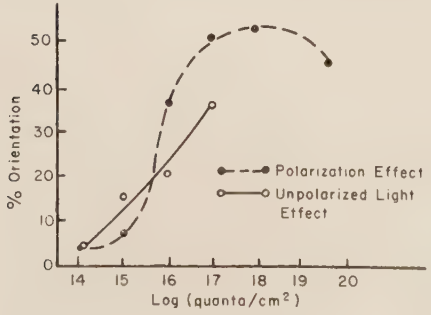

Fig. 4.—Phototropic response of *Pelvetia* eggs to horizontally directed white light.

some preformed asymmetric inclusion, such as the nucleus; it must arise in some more epigenetic manner.

It is also of interest to inquire further into the mechanism of the tropistic responses. First, let us consider the *subequatorial response* to unpolarized

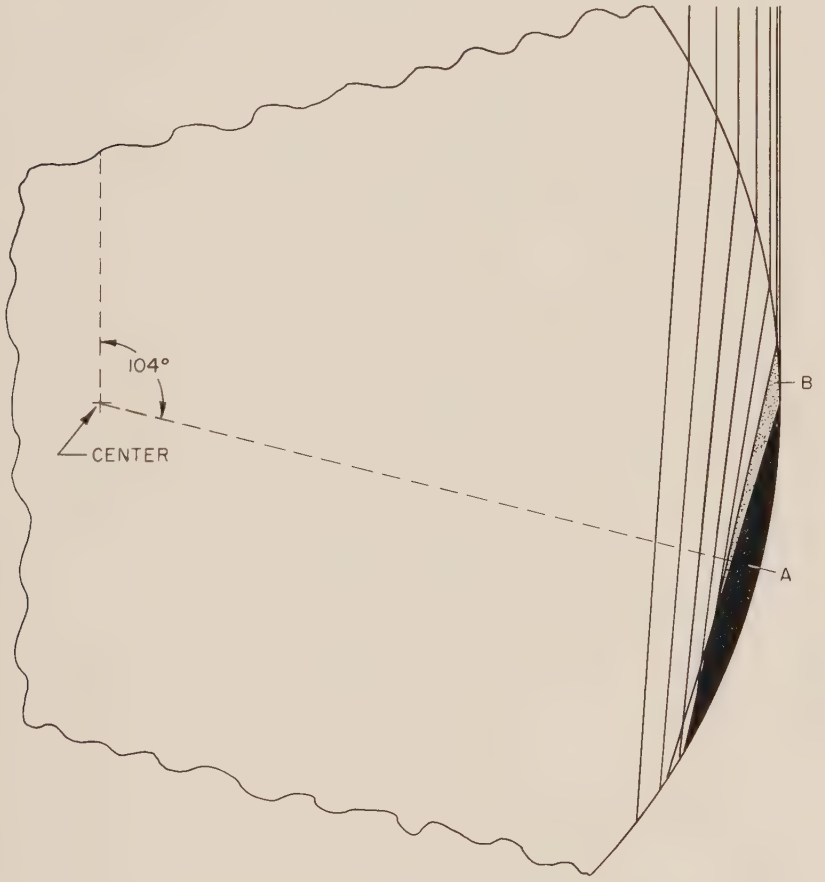

Fig. 5.—Model to show that a shallow subequatorial band in a unilaterally illuminated cell remains dark. Zone *A* is entirely by-passed, while zone *B* receives beams which have lost at least half their energy by external reflection. (Drawn for a spherical cell of refractive index, $n = 1.4$ immersed in a medium, such as sea water, for which $n = 1.34$).

light. Whitaker and Lowrance interpreted both responses as caused by an influence directing growth toward the rear pole; the subequatorial accumulations supposedly resulted from a more intense reorientation of partially determined primordia from the anterior hemisphere to the subequatorial

zone than from the latter region to the rear pole. If this were true, then the concentrations of outgrowths arising near the rear pole would be substantially above the uniform level, even in the most extreme cases; but there, they are, if anything, below this level. (See Table III, 10^{15} and 10^{16} quanta/cm^2; Table IV, illumination started 13 to 15 hours after fertilization.) Hence the subequatorial response must be considered a consequence of a truly subequatorial directive influence.

I can conceive of only one general hypothesis to explain such an influence: Growth is directed toward the subequatorial zone because its peripheral portions are the darkest region of the cell. Consider Fig. 5. Only scattered light reaches the by-passed band (A) at the zone's periphery and under it is a second shallow band (B) heavily shaded by reflection losses. This analysis likewise explains several other facts: (i) Spores of the mosses, *Neckera complanata* and *Amblystegium serpens* grow out at "90°" to unilateral and presumably unpolarized light of low intensities [14]. (Heitz's report is undocumented so it may well be that the response is actually *sub*equatorial.) (ii) The zygotes of four species of *Fucus* [16, 19] as well as spores of *Equisetum* [26] germinate equatorially in response to *bi*lateral illumination. (iii) The zygotes of *F. furcatus* (these results) and the spores of *Equisetum* [26] both grow out at 90° to the light directions in response to equatorial illumination. It should be emphasized that this analysis requires that the effective photoreceptors be concentrated in a surface layer. For those rays which are diverted from the by-passed zone by the cell's lens action are concentrated just beneath the surface (Fig. 5). If photoreceptors were as abundant in this subsurface region as in the more peripheral shell, then it would be most difficult to understand how the net photochemical action in the subequatorial zone could be lower than at the rear pole.

It likewise seems almost inescapable that when the *rear pole response* occurs, the most posterior region of the cell becomes effectually the darkest, but the mechanism of this shift is obscure. Photoreceptor molecules deeper in the cell may come into play, a subtle chloroplast movement may change the pattern of scattering and absorption, or the cell may integrate the incoming information by centering its response at the unique center of the darkest hemisphere instead of some indeterminate point of the darkest ring.

Now consider the mechanism of the polarotropic response. It is a variant of the subequatorial one. Hence in some manner the photoreceptor molecules in those meridians of the subequatorial zone lying parallel to the vibration plane must receive least light, How? Since Fresnel reflection is least effective at the cell surfaces in these meridians, *differential external reflection*

will cause a relatively greater illumination of the preferentially germinating regions and must be rejected as an explanation. Moreover, *differential internal reflection* cannot over-compensate for this effect because following its first internal reflection each ray has not only been weakened by its second reflection, by absorption and by scattering but traverses a region relatively far from the preferentially germinating one.

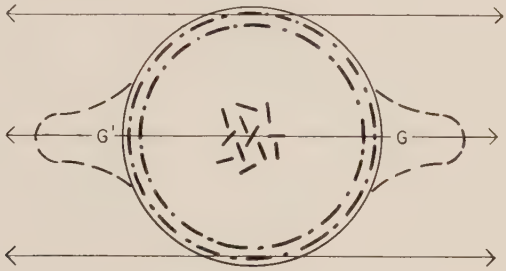

Fig. 6.—Model of egg to show that the photoreceptor molecules in the germinating regions (*G* and *G'*) will absorb least light if the photoreceptors are located near the cell surface and are periclinally oriented. Light enters perpendicular to plane of page, vibrating as shown by double-headed arrows. Dashes within egg represent the axes of maximum light absorption of photoreceptors; dots are photoreceptors lying perpendicular to plane of page; photoreceptors lying between polar and equatorial regions not indicated.

Two possibilities remain: (i) *Differential scattering:* Peripheral molecules in the meridian parallel to the vibration plane are open to substantially less light intracellularly scattered from rays vibrating perpendicular to the scattering plane than the corresponding molecules in the perpendicular meridian, and will therefore receive less scattered light. Moreover, since Latimer's recent studies [21] suggest that algal chloroplasts are remarkably effective scatterers, differential scattering cannot be lightly dismissed as too small an effect.

(ii) *Photoreceptor orientation:* This is the most attractive possibility, since the dichroic ratios of most dyes [20] are plainly large enough to explain the polarotropic response if these molecules were but oriented properly in the cell. Since the zygote looks and acts as though it were radially symmetrical, the only reference point for photoreceptor orientation is the cell surface. Hence these molecules, if they are oriented at all, must either tend to have their axes of maximum absorption arranged parallel to or perpendicular to the nearby cell surface. Examination of Fig. 6 shows that a parallel orientation is the requisite one. Acting together with the by-passing effect, it would cause the photoreceptor molecules in the germinating region to absorb the smallest fraction of the light passing through.

The one structure in which the arrangement of photoreceptor molecules is definitely known is the vertebrate rod. It is notable that this arrangement is analogous to that postulated above for the *Fucaceae* zygote. For the axes of maximum absorption in the rod lie in planes perpendicular to the rod and anisotropically within these planes [9, 28]; hence they lie periclinally, i.e. parallel to the nearby membranes [10, 30].

The only report of polarotropism, other than in the *Fucaceae* zygote, is that of Castle in the *Phycomyces* sporangiophore [5]. It is notable that this finding is most readily rationalized by invoking an arrangement of dichroic photoreceptors again analogous to that postulated for the *Fucaceae* zygote. Contrary to Castle's claim, differential reflection cannot explain the observation [17], while an alignment of photoreceptors in the direction of greatest wall strain can. In the case of the spherical *Fucaceae* zygotes this is simply the postulated periclinal one; in the case of the cylindrical sporangiophore, it is further restricted, being both periclinal and perpendicular to the cell's long axis [6].

Finally, it may be noted that the close-packed tubular membranes of the arthropod ommatidium are believed to define the region in which the photoreceptor molecules lie, and are so arranged that a periclinal arrangement of these photoreceptors could explain the capacity of the arthropod eye to detect the plane of vibration of light [11, 23, 35].

SUMMARY

The following picture is drawn from experiments on two members of the Fucaceae, *Fucus furcatus* and *Pelvetia fastigiata:*

1. In their "polarotropic" response to unidirectional illumination with plane polarized visible light, the zygotes tend to germinate in the plane of vibration, and "subequatorially" (from 90° to 135° away from the source). (Fig. 1.) Up to half the embryos so produced may be bipolar forms.

2. The tropistic response to similar unpolarized light is dual. Under some partially defined conditions it is subequatorial; under others, directly away from the source.

3. The polarotropic response and the subequatorial response to unpolarized light fade out over the same intensity range (Fig. 4).

4. The polarotropic action spectra (Fig. 3) belong to the phototropic group of spectra as found with other organisms (Table VI).

5. It is concluded that in all three tropistic responses to light, growth tends to occur where certain photoreceptor molecules absorb the least light.

6. It is suggested that under unpolarized illumination the subequatorial

and peripheral photoreceptors absorb least light primarily because they are by-passed by the cell's focusing action (Fig. 5), while under polarized illumination the subequatorial photoreceptors in the vibration plane absorb least light because they are periclinally oriented (Fig. 6).

I wish to thank Dr. Francis Haxo and Mr. James Snodgrass for the use of their equipment and facilities.

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A STUDY OF THE MECHANISM OF PINOCYTOSIS[1, 2]

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Received December 14, 1957

Lewis, from observations on macrophages [13] and sarcoma cells [14] in tissue culture, defined pinocytosis as the uptake of fluid droplets by cells. Since then it has been observed in a variety of cells. Mast and Doyle [18] observed pinocytosis in several species of amoebae. Observations with the electron microscope by Palade [19, 20], De Robertis and Bennett [6] suggest that pinocytosis may occur in mammalian cells within the animal. Reports by Gey and his associates [7, 8, 9] and Holter and Marshall [12] suggest that pinocytosis may be related to the formation of cell organelles such as mitochondria.

Previous works consisted of observations on the diversity of cell types which exhibit pinocytosis, or they were concerned with the intracellular role of the pinocytosis vacuole and its contents. However, pinocytosis is a mechanism by which cells transport solutes across the surface membrane, and therefore the method by which it is accomplished deserves further study. The work reported here was concerned for the most part with pinocytosis as a mechanism for the transport of solute across the cell membrane, and to a lesser extent to indicate what role it may have in the formation of cell organelles.

The large multinucleate amoeba, *Chaos chaos*, was chosen as the experimental cell because its environment could be easily controlled, and because its size facilitated handling and cytological observation. The experimental work consisted of three parts: (*a*) a study of morphology of amoebae during pinocytosis, (*b*) a study of the plasmalemma and its relation to the wall of the pinocytosis vacuole, and (*c*) a study of the experimental conditions under which amoebae exhibit pinocytosis.

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[1] This work was done in partial fulfilment of the degree of Master of Science in Anatomy at The University of Pennsylvania.

[2] This research was supported by Research Grant C-1957 from The National Cancer Institute of The National Institutes of Health, United States Public Health Service, U.S.A.

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MATERIALS AND METHODS

Methods utilized for studying living amoebae.—The amoebae were cultured in a solution of inorganic salts [22]. They were maintained by feedings of washed *Paramecium aurelia.*

A chamber was built of glass coverslips on a slide which allowed controlled compression of an amoeba. Solutions from a reservoir on one side of the chamber were drawn past the amoeba to a filter paper wick on the opposite side. Variation of the size of the evaporation surface of the wick permitted rough control over the rate of flow. The chamber compressed the amoeba to a thickness of ten to fifteen micra which was within the range of the 4 mm dark medium phase-contrast objective [43]. Most of the observations on living amoebae were made with the phase-contrast microscope.

In order to test the effect of different environmental conditions a sequence of perfusion fluids was used. First an amoeba was perfused with ion-free water, second with a test solution, and third with the culture solution. The compositions of the test solutions (Table I) in the second step were varied to explore the effects of solute, time of exposure, concentration, and pH on the amoeba and to find the most efficient conditions for stimulating pinocytosis.

The test solutions (Table I) of salts and proteins were made up in ion-free water. The proteins used were normal dog, rabbit, and chicken γ-globulin, rabbit serum albumin, ovalbumin, and β-lactoglobulin. The γ-globulins and serum albumin were dialyzed against several changes of ion-free water. The ovalbumin and β-lactoglobulin were prepared by dissolving crystalline protein in ion-free water. The solutions of γ-globulins in saline were prepared by dialyzing rabbit and chicken γ-globulin against several changes of a solution of NaCl (0.01 *M*). The versene solution was prepared by dissolving disodium versenate (0.001 *M*) in ion-free water and titrating it with dilute NaOH to a pH of 6.3.

TABLE I.

Test solutions	Concentration	Total trials	Pinocytosis +	Pinocytosis −
Proteins	1–2 % approx.	42	25	17
Sodium chloride	0.01 *M*	7	7	0
Potassium chloride	0.01 *M*	6	6	0
Versene	0.001 *M*	32	19	13
Calcium chloride	0.001 *M*	11	3	8
Culture solution		23	0	23
Ion-free water		46	1	45
γ-globulin in 0.01 *M* NaCl	2 %	17	14	3

In each trial one or more amoebae were observed in the test solution. The (+) sign under pinocytosis indicates the number of trials in which the amoebae were observed to pinocytose in the test solution.

Methods utilized for studying fixed amoebae.—Amoebae were arrested in different stages of pinocytosis by freeze-drying. Before being frozen these amoebae were exposed to the same sequence of solutions as the living amoebae which were observed in the compression chamber. The time of the final wash was varied between one minute and 100 minutes in order to follow as closely as possible the progressive stages of pinocytosis. The amoebae were then pipetted into liquid propane kept at −170°C by a liquid air bath. After freezing, they were dried and embedded in diethylene-glycol distearate *in vacuo*. They were sectioned at one micron by a glass knife onto a trough filled with 4 per cent formaldehyde. The sections were placed on a slide without adhesive, dried at 40°C, then cleared in xylene. Some sections were stained before mounting. Stained and unstained sections were mounted in U.S.P. light mineral oil of Refractive Index 1.45 and examined with the phase-contrast, light, or fluorescense microscopes.

Staining of sections.—Periodic Acid Schiff (PAS) staining of the plasmalemma [1, 21] was used to follow the changes in the plasmalemma during and after pinocytosis.

In order to trace the uptake of solutes some amoebae were treated with fluorescein-labeled rabbit γ-globulin solutions before being frozen. Under the fluorescence microscope, sections of these amoebae showed a fluorescence of the plasmalemma and of the contents of numerous vacuoles. Because of the possibility that this staining was due to fluorescein derivatives not bound to the test proteins, a second method of tracing proteins was employed. Antibodies were prepared in rabbits and chickens against unlabeled proteins which were to be used in test solutions. The γ-globulin fractions of the sera of these animals were conjugated with fluorescein according to the method of Coons and Kaplan [5] and Marshall [16, 17]. Complete control of non-specific staining was possible by this method.

For the antibody staining procedure amoebae were treated with an unlabeled test protein before freezing. After freeze-drying these amoebae were sectioned, cleared in zylene, and brought to 25 per cent glycerol through an alcohol series. A drop of antibody conjugate reagent prepared in 25 per cent glycerol was placed over the sections. The sections were left to stain overnight in a refrigerator kept at 5°C. The sections were then washed in 25 per cent glycerol to remove the excess antibody reagent. Specificity of the staining was demonstrated by comparing sections stained with an antibody conjugate specific for the test protein to alternate sections stained with normal γ-globulin conjugate. All antibody conjugates were tested for non-specific staining against sections of control amoebae.

EXPERIMENTAL RESULTS

Morphology of living amoebae during pinocytosis.—The surface of *Chaos chaos* as observed in the compression chamber in phase contrast is normally smooth except in the tail area where folds are common. The tip of an advancing pseudopod is hyaline, and a gel sheet can be seen immediately under the plasmalemma. This gel sheet breaks away from the membrane in a series of jerks, as plasmasol flows between it and the plasmalemma. A

new gel sheet forms against the membrane from the plasmasol, and this gel sheet in turn breaks away from the plasmalemma as a new flow of plasmasol occurs. This stepwise process continues as long as the pseudopod advances. Under favorable circumstances one can see a series of displaced gel sheets spaced at intervals behind the advancing tip. These gel sheets are increasingly penetrated by channels of the advancing plasmasol, and eventually disappear, presumably by complete solution. These events, which correspond to the observations made by Goldacre [10], are modified during pinocytosis.

Immediately prior to pinocytosis the plasmalemma becomes finely wrinkled over the whole cell surface. Pseudopods in the process of growing out stop, and then begin to retract. During this retraction some points on the plasmalemma are drawn down into the cell. Again plasmasol slowly begins to flow, pressing against the plasmalemma, but now the plasmalemma is not lifted uniformly from the underlying gel. Some of the points of attachment of the plasmalemma to the gel resist separation. The attachment of these points seems relatively strong, for during this phase of pseudopod advancement, tunnels or invaginations of the plasmalemma deep into the cytoplasm are formed. These invaginations appear to be drawn back into the cytoplasm by gel retraction as much as they are built up by the advancing edge of the cell. Small droplets are pulled off the tips of these tunnels or complete sections of them are detached and form coiled tube-shaped inclusions (Fig. 1). The points where the plasmalemma is drawn back into the cell may be so close and numerous that entire areas of the membrane may be invaginated. These invaginations form large, highly irregular vacuoles (Fig. 2).

Pinocytosis was most commonly observed in the tail area. In some solutions tested (Table I) pinocytosis occurred over the entire cell surface. Usually large irregular vacuoles were taken up by amoebae exposed to test solutions which consistently caused pinocytosis over the entire cell surface. No attempt was made to determine the fate of these vacuoles, but several times large vaculoes were seen to break up into smaller ones.

Morphology of amoebae fixed during pinocytosis.—The Periodic Acid-Schiff technique stained the membranes of some vacuoles. These were tentatively identified as pinocytosis droplets because their walls stained similarly to the plasmalemma, and because these vacuoles were similar in size and shape to the pinocytosis vacuoles which were observed in living amoebae. More conclusive evidence for the identification of the pinocytosis vacuoles comes from observations on amoebae treated with both labeled and non-labeled protein solutions. The results were the same when labeled test proteins were fed to amoebae as when unlabeled test proteins were fed and

later detected by antibody staining. Many fluorescent vacuoles were visible in sections of these amoebae ranging in size and shape from droplets less than one micron in diameter to large irregular vacuoles unique to cells which have exhibited pinocytosis (Figs. 3 and 4). The antibody staining technique showed that both the plasmalemma and the wall of the pinocytosis vacuole had adsorbed protein (Figs. 5 and 6).

Amoebae treated with unlabeled protein before freezing were used to correlate PAS and fluorescent antibody staining. Alternate sections of these amoebae were stained either by PAS or fluorescent antibody. The staining pattern from the two techniques was identical with respect to the plasmalemma and pinocytosis vacuole walls.

In many stained sections of amoebae it was observed that the plasmalemma appeared to split into two layers (Fig. 7). The outer layer was more heavily stained both by PAS and by fluorescent antibody (Fig. 8). The thickness of the PAS staining outer layer increased several times in the presence of versene (0.001 *M*) or sodium chloride (0.01 *M*) (Fig. 9). The apparent swelling of the plasmalemma occurred within five minutes after the amoebae were placed in the salt solution.

Experimental conditions causing pinocytosis. Living amoebae.—In previous work [18, 3] and in all the experiments reported here pinocytosis was found to be a discontinuous phenomenon. The amoebae were stimulated to exhibit pinocytosis by being introduced into an active solution from an inactive one. Pinocytosis lasted about fifteen minutes, slowly decreasing after an initial burst. Before further pinocytosis could be stimulated, the cells had to be

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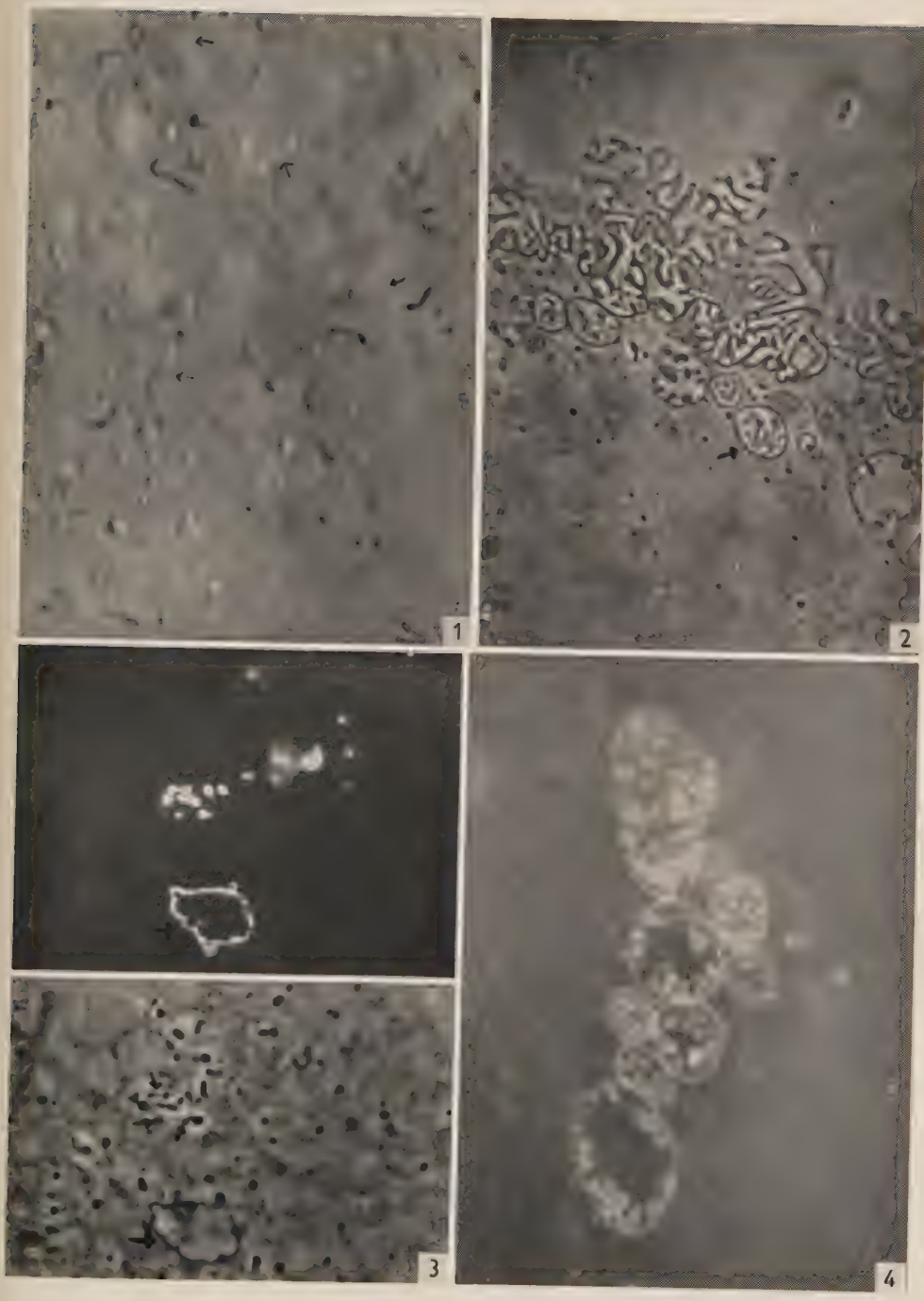
All the phase-contrast photomicrographs were taken on Kodak M plates using four second exposures. All fluoresenct photomicrographs were taken on Kodak 103a-g spectrographic plates. Exposures were from 10 to 20 minutes.

Fig. 1.—A phase contrast photomicrograph of a living amoeba taken in the plane directly beneath the plasmalemma. Several coiled pinocytosis tubules are indicated by arrows. A contractile vacuole surrounded by mitochondria is shown in the lower left. Numerous crystals are visible. × 1500.

Fig. 2.—Formation of large irregular pinocytosis vacuoles are shown in a photomicrograph taken with a phase-contrast microscope. × 1200.

Fig. 3.—A comparison of phase-contrast (below) to fluorescence (above) photomicrography of the same section. One large and numerous small pinocytosis vacuoles are indicated by arrows. Note the similarity of pinocytosis droplets in the fluorescence photomicrograph to structures indistinguishable from mitochondria in the phase-contrast photomicrograph. × 2000.

Fig. 4.—A fluorescence photomicrograph of pinocytosis vacuoles. Many infoldings of the vacuole membranes are shown. × 2000.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washed with ion-free water or with culture solution. Upon returning the amoebae to an active solution the pinocytosis cycle was repeated. When one active agent was followed by a second, it was found to be ineffective in initiating a full pinocytosis cycle although it appeared to prolong the decreasing phase.

Amoebae were tested with various solutions in the continuous flow compression chamber. The results in Table I show that proteins, versene, potassium chloride and sodium chloride were active. Sodium and potassium chloride solutions showed the most consistent activity. Among the proteins tried, γ-globulins were more consistently active than the other proteins.

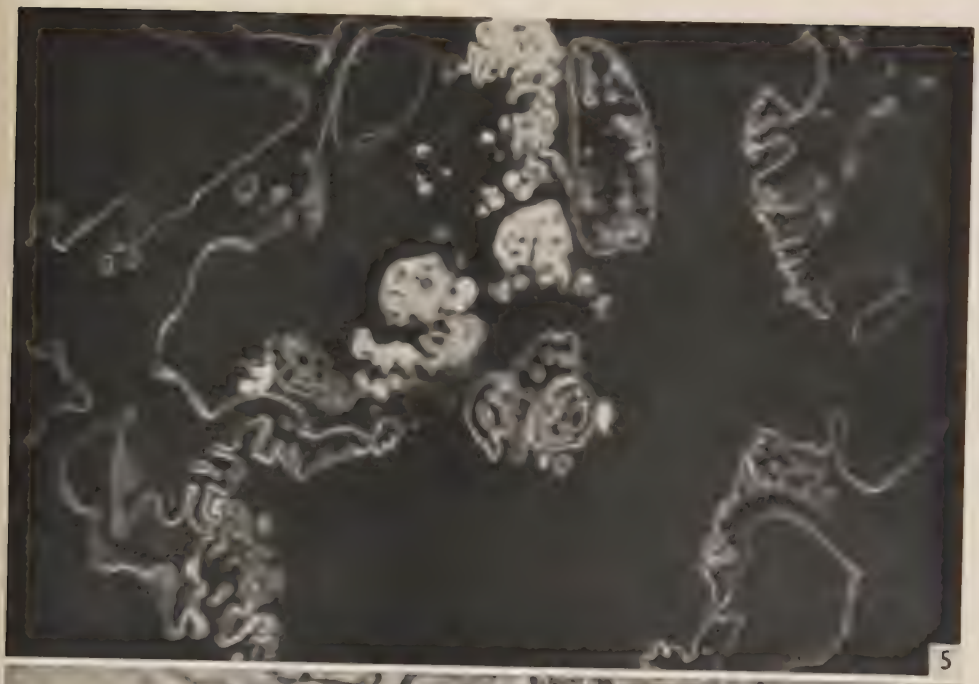
Fixed and sectioned amoebae.—The effects of pH, concentration, exposure time and temperature were tested by freeze-drying experimental amoebae. Since each test involved eight or ten amoebae, all of which were sectioned together, estimates of the number of vacuoles and density of the surface coating were possible.

Pinocytosis was found to be independent of the pH over a range from pH 6.5 to pH 8.0 when sodium chloride was the test solution.

To test the effect of concentration on pinocytosis and on adsorption of protein to the plasmalemma, dilutions of the test solution were used. γ-Globulin (2 per cent) in sodium chloride (0.01 *M*) solution was diluted by 1/2, 1/10, and 1/50. The solution at full strength caused pinocytosis but none of the dilutions were active. The adsorption of protein on the plasmalemma appeared to decrease only slightly at 1/2 and 1/10 dilution, and remained faintly visible at 1/50 dilution. In this experiment amoebae were exposed to all the test dilutions for ten minutes. Amoebae exposed for 100 minutes appeared to contain fewer vacuoles than amoebae exposed for ten minutes.

In order to test the importance of active streaming on pinocytosis, amoebae after being exposed to a test solution were placed in a 5°C wash for ten to one hundred minutes, then frozen. The amoebae placed in the cold wash ceased streaming and rounded up while the amoebae washed at room temperature continued active streaming. Sections of the amoebae which were washed in the cold showed a great increase in the amount of pinocytosis over control amoebae washed at room temperature.

Figs. 5 and 6.—A comparison of a phase-contrast (Fig. 6) to a fluorescence (Fig. 5) photomicrograph. Numerous pinocytosis vacuoles can be identified by their fluorescent walls. The coiled tube-like pinocytosis vacuoles are resolved by phase-contrast into membrane-enclosed sacs. ×2000.



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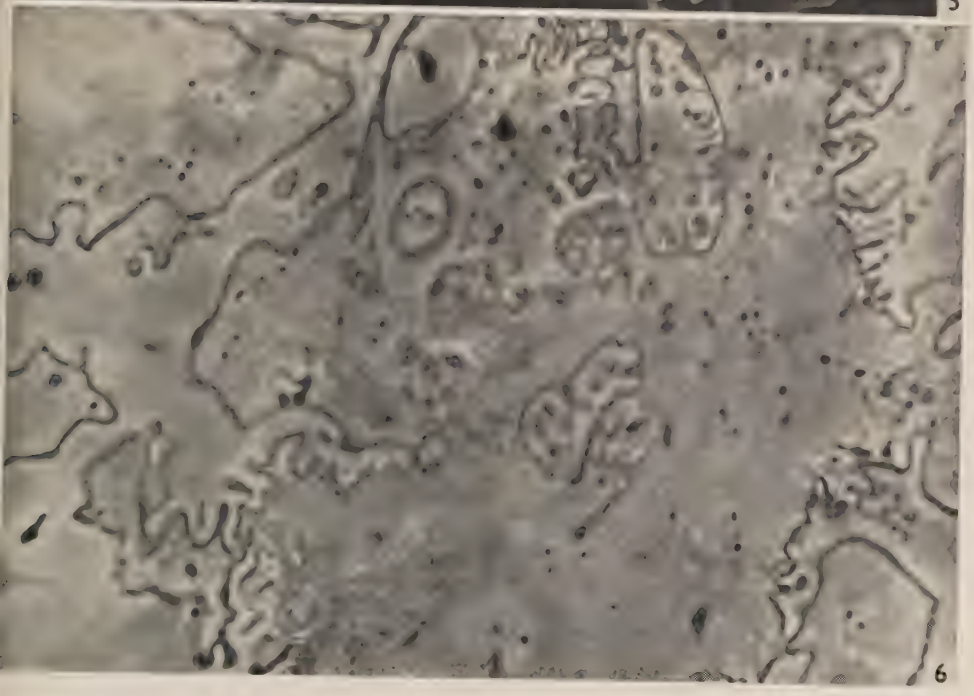

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DISCUSSION AND CONCLUSIONS

Fate of the pinocytosis droplets.—This study was concerned primarily with the mechanism of formation of pinocytosis droplets, but equally as interesting as the mechanism of their formation is the fate of the pinocytosis vacuoles and their membranes. The findings of this study from PAS staining and membrane adsorption of protein show that the plasmalemma is the source of the membranes of pinocytosis vacuoles. There is evidence that the plasmalemma is altered after its incorporation into the pinocytosis vacuole. Chapman-Andresen and Holter [3] reported that glucose labeled with ^{14}C is quickly lost to the cytoplasm from the pinocytosis vacuole, although the intact plasmalemma is relatively impermeable to glucose. Labeled glucose ingested with albumin during pinocytosis was utilized by the cell, and ^{14}C was incorporated into many cell organelles.

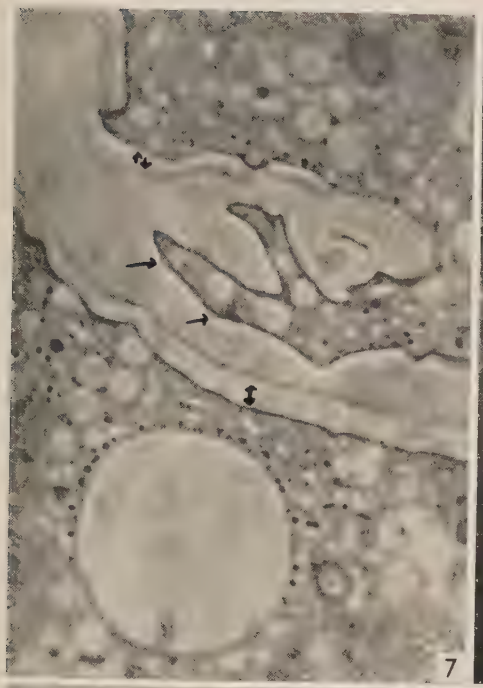
Gey [7] suggested that the membrane-bound structures seen in mammalian cells by electron microscopy may have a surface origin. Bennett [2] has recently discussed some theoretical functions of a membrane incorporation concept. The pinocytosis vacuoles often appear as highly convoluted tubules (Figs. 5 and 6) resembling the intracellular membranes of mammalian cells, although the scale of size is much larger in amoebae.

There is evidence which suggests that a close relationship exists between pinocytosis vacuoles and mitochondria. Holter and Marshall [12] fed amoebae protein labeled with fluorescein. When these amoebae were centrifuged three days later, fluorescent granules were detected in the mitochondria layer. Gey *et al.* [8] observed fusion of pinocytosis droplets with mitochondria in tissue culture cells. They also reported that pinocytosis droplets evolved into forms which could not be differentiated from mitochondria. The phase-contrast and fluorescence findings of this study show that pinocytosis droplets are often identical in size, shape, and density with mitochondria in amoebae (Fig. 3). Study by fluorescence and electron microscopy may further establish the relationship between mitochondria, intracellular membranes, and pinocytosis vacuoles.

Fig. 7.—A phase-contrast photomicrograph of a section of two pseudopods of an amoeba showing areas where the membrane is split. This splitting is sometimes slight, or complete separation of the plasmalemma into two layers can occur. Examples of both are indicated by arrows. × 1000.

Fig. 8.—A fluorescent photomicrograph which shows the staining of the split plasmalemma. The outer layer is more heavily stained in some areas. × 1000.

Fig. 9.—A phase-contrast photomicrograph of a section of an amoeba which was treated with versene before freezing. The surface of the plasmalemma appears to be swollen. × 2500.



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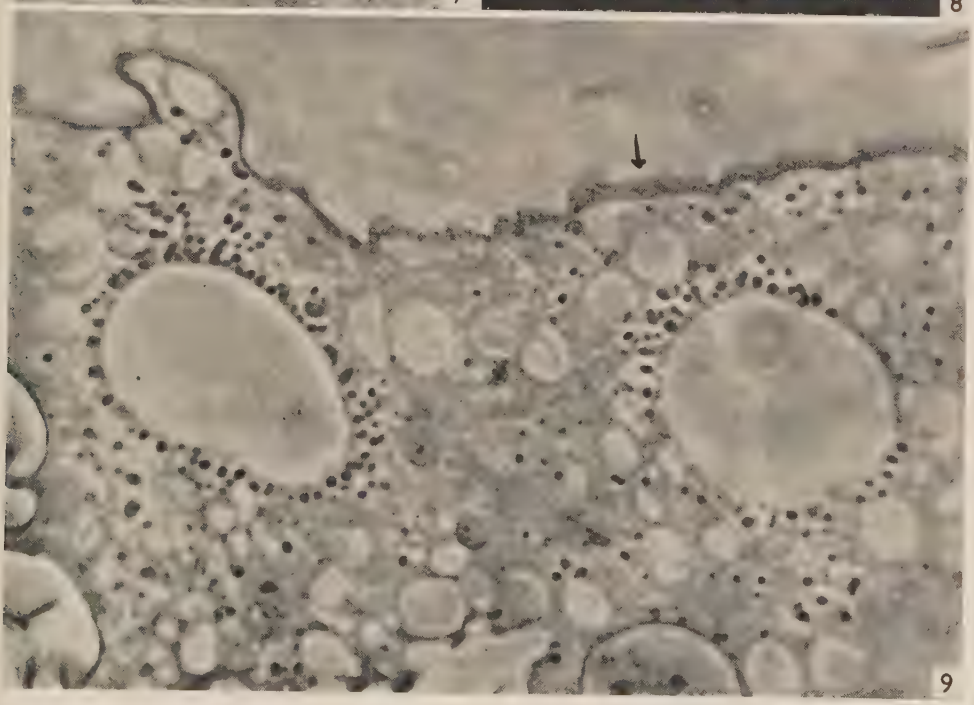

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Mechanism of pinocytosis.—There is evidence that the mechanism of pinocytosis is related to changes in the cell surface area. Pinocytosis is most common in the tail area, which is postulated to be a site of membrane reclamation [10, 15]. When an amoeba is rounded up from an extended form there must be a decrease in surface area. Protein-treated amoebae subsequently washed and rounded up in the cold show many more pinocytosis vacuoles than amoebae washed at room temperature. These simple observations suggest that the mechanism of pinocytosis depends on a direct physical change in membrane tension and area.

From observations on living amoebae during pseudopod formation, it is apparent that the plasmalemma is freed from the underlying gel in a series of jerks, as the plasmasol is forced between these two. One can see that there are points in the attachment of the plasmalemma to the gel sheet that resist separation more strongly than the surrounding area. These points of attachment appear to be under tension as the plasmalemma is forced outward by the plasmasol pressure. Under ordinary conditions such points of attachment soon snap apart, and the slight dimple on the membrane caused by the attachment is thus erased. Presumably, detachment occurs when the tension of the membrane increases sufficiently to break this weak bond.

A fine wrinkling of the membrane was always observed prior to pinocytosis when amoebae were treated with an active agent. It seems logical to infer that membrane tension has dropped markedly in such instances, and that the membrane tension which normally breaks the bonds of attachment between the plasmalemma and underlying gel is now inadequate. As a result the plasmalemma is drawn down into the cytoplasm at such points of attachment. It appears to be pulled in deeper by the plasmasol flowing forward into the pseudopod and pushing the plasmalemma ahead of it.

Three general mechanisms which could decrease the tension of the plasmalemma may be outlined.

(*a*) The most direct is the effect on surface tension of adsorption of charged molecules, such as salts and proteins.

(*b*) If one considers the plasmalemma not as a simple interface but as a complex structure, a second mechanism is suggested. Agents which are adsorbed may affect the internal bonding and structural rigidity of the plasmalemma. This possibility is suggested by the observations that the plasmalemma is indeed structurally complex and that it undergoes swelling in salt and versene solutions which induce pinocytosis.

(*c*) A more remote mechanism is an indirect effect of the solutions on the

mechanism by which the amoeba normally alters its surface membrane area. Since the plasmalemma may be continuously exchanging with a component of the cytoplasm, an agent which interfered with the reclamation of the plasmalemma might cause a decrease in membrane tension.

Whatever may be said of these hypotheses, it has been shown that all charged solutes which have been tested have proved to stimulate pinocytosis in amoebae. Similar or higher concentrations of non-charged solutes such as "sugar" [18], or glucose and starch [3] have failed to stimulate pinocytosis in amoebae. Mast and Doyle [18] compared "sugar" (presumably sucrose) and calcium gluconate; they found that the latter would cause pinocytosis while sucrose would not, even in higher concentrations. Their observations and those reported in this study strongly suggest that ionic charge of the solute is the essential factor in determining whether or not the solute will induce pinocytosis in amoebae. Ionic charge is a major factor in the adsorption properties of a molecule.

Evidence for this hypothesis can be derived from the work of Schumaker [23] who kindly permitted the author to see his results prior to publication. Schumaker measured the rate at which ribonuclease and cytochrome *c* tagged with ^{131}I were taken up from solution by *Amoeba proteus*. He showed that there is an initial and reversible phase to protein uptake which lasted approximately five minutes, and which is independent of the cell's energy sources. The protein in this initial phase of uptake sometimes amounted to the protein in a volume of solution twenty-five times that of the amoeba. Schumaker postulated that this reversible phase of protein uptake consisted of adsorption of protein on the cell surface. His plots of protein uptake against concentration show a sudden increase in uptake above a certain concentration of protein. Schumaker postulated that this sudden increase in uptake was due to the unmasking of additional adsorption sites on the membrane. This is indirect evidence for the hypothesis that adsorption of charged ions weakens the internal bonds of the plasmalemma. The results of this study and that of Schumaker show that the surface binding of proteins provides amoebae with a method of concentrating external solutes with only the expenditure of the metabolic energy necessary to replace its membrane.

Mast and Doyle [18] suggested that pinocytosis is induced by an osmotic loss of water in a hypertonic medium. Their measurements of volume did reveal a shrinkage of amoebae in solutions which induce pinocytosis. However, two objections may be raised to the concept that pinocytosis is a simple

compensatory mechanism designed to restore cell volume or water. The first, derived from their own observations, is that sucrose will cause a loss in cell volume but will not induce pinocytosis. The second is that the concept has a teleological basis, yet even on teleological grounds, it is difficult to see how uptake of hypertonic solutions could have survival value.

Chapman-Andresen and Prescott [4] reported many interesting observations of pinocytosis in amoebae but did not attempt to interpret the phenomenon, except to suggest that "pinocytosis may perform a double function, a nutritional one with the ingestion of protein and a defense against a concentrated ionic environment with the uptake of water and ions".

It seems consistent with the experimental results to conclude that adsorption of charged ions to the membrane induces pinocytosis by decreasing the tension and weakening the internal structure of the plasmalemma. Due to the decrease tension on the membrane, the normal gel contraction pulls the point of plasmalemma "attachment" into the cytoplasm. Because of the weakening of the internal structure of the plasmalemma, the tunnels so formed are separated from their attachment to the plasmalemma and are often broken up into small droplets.

SUMMARY

1. The morphology of living amoebae during pinocytosis is described. These observations are correlated with observations on stained sections of amoebae fixed during pinocytosis.

2. Immediately before pinocytosis, wrinkling of the plasmalemma was observed. Wrinkling was followed by invagination of the plasmalemma which resulted in tunnels into the cytoplasm. These tunnels were then separated from the plasmalemma and were often broken up into droplets.

3. PAS and antibody staining procedures confirmed the observation that the pinocytosis vacuole membrane is derived from the plasmalemma.

4. All solutions of charged molecules which were tested, including many proteins and salts, were found to induce pinocytosis. Their activity was dependent to some extent on their concentration.

5. By fluorescence labeling techniques it was demonstrated that proteins are adsorbed to the plasmalemma. Salt solutions which caused pinocytosis were shown to cause a swelling of the outer coat of the plasmalemma.

6. A mechanism for pinocytosis is suggested which relates adsorption of charged solutes on the plasmalemma to a decrease in the tension and the structural rigidity of the membrane. The weakened membrane is drawn

down into the cytoplasm by its "points of attachment" to the contractile plasmagel system. These invaginations become detached from the membrane and freed in the cytoplasm as pinocytosis droplets or vacuoles.

The author would like to thank Dr. John M. Marshall for all the help received from him during the conduct of this work.

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UPTAKE OF PROTEIN FROM SOLUTION BY *AMOEBA PROTEUS*

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Received December 15, 1957

The uptake of protein molecules from solution by the amoeba has been studied by Holter and Marshall [8] who measured the amount and localization of protein tagged with a fluorescent dye by direct microscopic measurements upon living and squash preparations of amoebae. These authors found that the giant amoeba, *Chaos chaos*, was able to take up in one hour the quantity of protein contained in a volume of solution equal to 30 per cent of the volume of the amoeba. The dyed protein appeared to be localized in droplets or vacuoles inside the amoeba, and these authors believed that the protein was ingested by a process called pinocytosis, a phenomenon which had been originally observed in mammalian cells by Lewis [9] and later in the amoeba by Mast and Doyle [11].

This work was extended by Chapman-Andresen and Holter [4] and Chapman-Andresen and Prescott [5] who studied the amount and fate of radioactively labelled glucose and methionine taken up by the amoeba and then metabolized. Since glucose does not rapidly diffuse into the amoeba, protein was added to the solution to cause pinocytosis.

These authors found that under this condition glucose did indeed enter the amoeba where it was utilized metabolically with the production of radioactive CO_2.

In the present study, the kinetics of the uptake of two proteins, ribonuclease (RNase) and cytochrome *c*, were measured as a function of time, concentration, temperature, pH, metabolic inhibitors, etc. The data obtained allow a detailed hypothesis to be presented for the uptake of protein from solution by the amoeba.

The protein RNase was used for most of the studies reported herein because it has been found to penetrate into many different cells where it causes metabolic and chromosomal damage. Brachet [2] has studied the

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effects of RNase solutions upon amoebae and finds that within 5 or 10 minutes the basophilia begins to decrease, especially in the nucleoli, and within 15 minutes the RNA content has dropped 10 per cent. Prolonged treatment causes the RNA content to drop 20 to 45 per cent and the incorporation of radioactive phenylalanine to be inhibited 80 to 90 per cent. These observations indicate that the enzyme penetrates the outer membrane of the amoeba and that it may reach the nucleoli within the space of a few minutes. The penetration of RNase has been confirmed by radioautography [1].

A detailed study of the kinetics of uptake of RNase may therefore be of aid in the elucidation of the metabolic disturbances which are caused by the penetration of this protein into living cells.

MATERIALS AND METHODS

Preparation of radioactive protein.—The following procedure is a modification of that employed by Francis *et al.* [6]: Approximately 20 mg of the A fraction [7] of chromatographically fractionated Armour RNase (kindly supplied by Dr. L. Ledoux) or cytochrome *c* (horse heart, Sigma) were added to 8.5 cc doubly distilled water in a small Erlenmeyer flask. To this solution was added 0.4 ml of 5 *N* NH_4OH. Immediately 1 ml of a carrier free $Na^{131}I$ solution containing approximately 1.5 millicuries ^{131}I was added to this solution. Finally, 0.8 ml of a 0.01 *M* KI solution, which had equilibrated overnight with finely ground I_2 and was then filtered twice to remove the excess I_2, were added drop by drop to the Erlenmeyer. The mixture (well corked) was kept at room temperature (20.8°C) for 15 minutes. Then 1.6 ml 1 *N* acetic acid were added, and the solution transferred to a dialysis sac. The Erlenmeyer was washed with 2 ml H_2O and this wash added to the dialysis sac. The solution was dialyzed against 3 changes (1000 ml each) of 0.9 per cent NaCl and then 4 changes of distilled water. By the 6th dialysis, the count had dropped to 32 cpm/ml for the solution outside the dialysis sac. After the 7th dialysis, the solution inside the sac was removed. The solution was then centrifuged at 25,000 rpm for 30 minutes in the Spinco Model L anglehead centrifuge. The supernatant was decanted and phosphate buffer added to make 0.003 *M* phosphate pH 6.2. The spectrum of the solution and the activity were measured. The specific activity of the RNase solution was $3.74 \times 10^{+6}$ cpm/milligram.

Treatment of amoeba (general).—*Amoeba proteus* was used for all the experiments reported in this communication.

Approximately 50 amoebae (starved) are transferred by pipette to a conical-bottom centrifuge tube. The tube is then spun "a few turns of the crank" in a hand centrifuge, causing the amoebae to pellet on the bottom of the tube. The excess liquid is removed by pipette, leaving the amoebae in a few λ (0.001 ml) of solution. Then about 1 ml of the radioactive ribonuclease solution is added rapidly, dispersing the pellet and scattering the amoebae throughout the solution. The tube is then placed at an acute angle with the table so that the amoebae will remain apart as they settle. After the appropriate time, temperature, pH, etc., the amoebae are

spun to the bottom of the tube (20 turns of the crank at a fairly good clip), the solution poured off rapidly, and a wash solution added. The amoebae are washed in this manner 4 *more* times. After the 4th wash, the amoebae are picked up with a pipette and transferred to a small dish containing about 3 ml of liquid. The amoeba are gently washed in this dish, and then picked up with the pipette and transferred to a second dish. Again the amoebae are washed and transferred to a third dish. This procedure is performed beneath a binocular microscope and all hairs and dirt are eliminated. The amoebae are then transferred to a plaque (for counting in the Geiger counter) with about 0.05 ml or less liquid from the last wash. The number of amoebae are counted, and finally all the excess liquid is removed from the plaque with a very fine capillary pipette. The plaque is then dried and the radioactivity determined. This total washing procedure takes between 10 and 15 minutes, and unless otherwise stated all samples were on the plaques within this time interval.

Measurement of enzymatic activity.—Two hundred amoebae were used for each measurement. After treatment with RNase and washing, the amoebae were dissolved in 0.1 ml of *N* NaOH for 15 minutes, extracted with 0.2 ml of *N* H_2SO_4 at 4°C for an hour, and neutralized by a second addition of 0.1 ml of *N* NaOH. The pH was adjusted to pH 4 by the addition of 0.2 ml 0.01 *N* acetate buffer and the preparation was centrifuged. To 0.5 ml of the supernatant was added 0.2 ml 1/15 *M* Na_2HPO_4. To this solution 0.5 ml of a 2 mg/ml solution of yeast ribonucleic acid in 0.01 *M* acetate buffer, pH 5.0 (Schwarz, twice precipitated with perchloric acid-ethanol as indicated below and dialyzed) was added and mixed well. The solution was then incubated at 37.5°C, and at 2 minutes and 30 minutes after the start of incubation 0.2 ml aliquots were removed and precipitated in 0.4 ml perchloric acid-ethanol (14 ml 70 per cent PCA in 86 ml absolute ethanol) at −15°C for 15 minutes. The solutions were then centrifuged and after centrifugation, 0.2 ml of the supernatant was removed, diluted to 2 ml and the optical density at 260 mμ and 300 mμ measured. Control experiments were performed using untreated amoebae with 0, 0.5, 1.0 and 1.5 γ RNase added to the amoebae just prior to dissolution in the NaOH. (The unit γ is used to denote 10^{-6}.) The increase in the corrected optical density (OD^{260} mμ–OD^{300} mμ) between the 2 minute and the 30 minute aliquots was calculated. From this figure was then subtracted the small increase in optical density found when no RNase was added to the amoebae. The remaining increase in optical density was found to be directly proportional to the ribonuclease concentration, and 1 γ of RNase corresponded to an increase of 0.63 optical density units.

EXPERIMENTAL

Unless otherwise indicated, protein solutions used in the experiments described below were buffered with a 0.003 *M* potassium and sodium phosphate buffer at pH 6.2. In order to demonstrate that the enzymatic activity of RNase is not a major factor in determining the uptake of this protein by the amoeba, parallel studies of the uptake were performed using solutions of RNase and solutions of cytochrome *c* of the same concentration. The results of these studies are reported in Fig. 1 in which the quantity of the enzyme

taken up by the amoebae is plotted as a function of time. It is seen that the agreement between the uptake curves is very good at the lower concentration employed, and is sufficiently satisfactory at the higher concentration to rule out the hypothesis that the enzymatic activity of RNase is a predominating factor in the uptake of this protein by the amoeba.

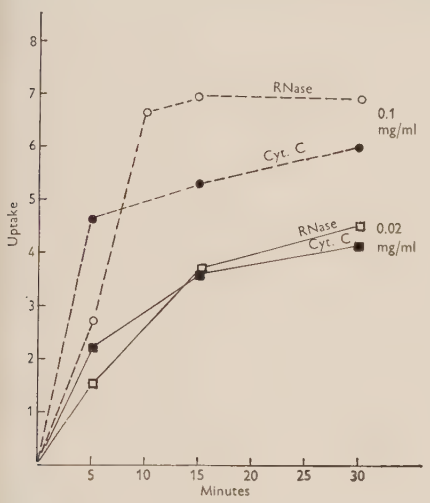

Fig. 1.

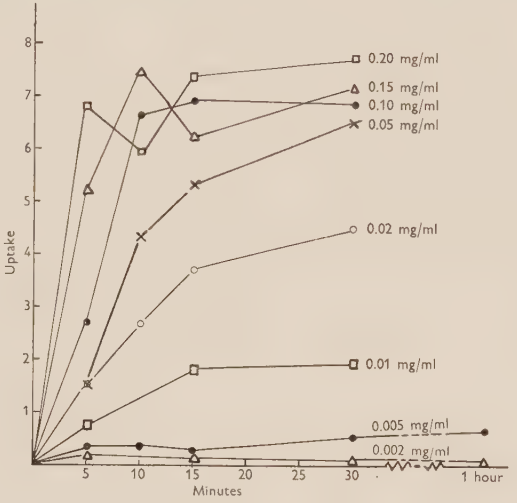

Fig. 2.

Fig. 1.—Uptake of RNase and cytochrome *c* from solution by *Amoeba proteus*. The abscissa is time in minutes; the ordinate is the quantity of ribonuclease taken up in units of 10^{-6} g/1000 amoeba. The concentrations of the external protein solutions are indicated on the graph.

Fig. 2.—Uptake of RNase by the amoeba as a function of the external protein concentration. The abscissa and ordinate of this figure are the same as for Fig. 1.

A series of experiments were performed using different concentrations of the radioactive ribonuclease preparation to follow the uptake of RNase as a function of time in the amoeba. The experiments were all performed at room temperature which varied between 19 and 22°C. The results of these experiments are given in Fig. 2. It is interesting to note the two distinct saturations or equilibrium effects which are apparent in the data presented in this figure: (1) At each of the various concentrations employed the uptake curve begins to level off and approach a plateau after about 30 minutes of treatment. (2) At the higher concentrations used these final plateau values are not much different and apparently a saturation point is reached at about the level of 8 γ RNase per 1000 amoeba.

Choosing a particular time of treatment (15 min.) a cross-plot of the data in Fig. 2 is given in Fig. 3 which also includes additional determinations made

at relatively low concentrations of the enzyme. Here again there are two distinct points of interest. (1) In the region of concentrations greater than 0.05 mg/ml, the 15-minute determinations approximately represent the final quantity of ribonuclease taken up by the amoeba, and it can be clearly seen that at these high concentrations amoebae become saturated with ribonu-

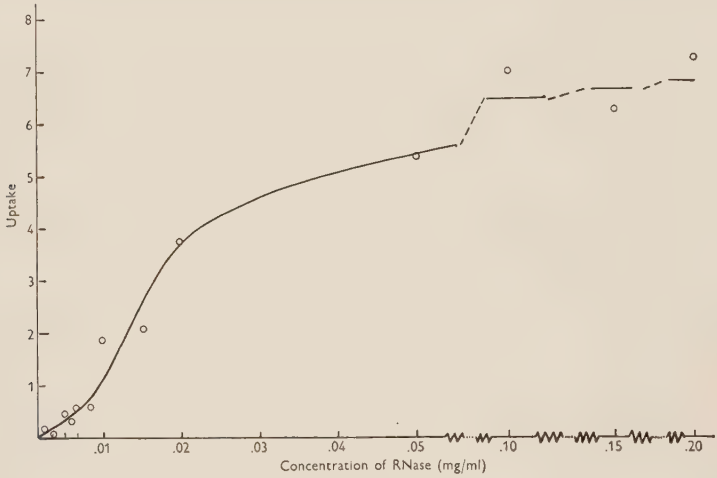

Fig. 3.—Uptake of RNase after 15 minutes of treatment as a function of the external concentration of protein. The abscissa is the external concentration of protein.

clease. (2) In the region of concentrations less than 0.02 mg/ml the 15-minute determinations are approximately proportional to the initial rate of uptake of RNase by the amoeba (see Fig. 2). Therefore, the first part of the curve in Fig. 3 can be roughly considered as a plot of the initial velocity of the reaction. It can be seen from Fig. 3 that this initial velocity of uptake of ribonuclease is not a linear function of the external concentration of the enzyme, but instead bends upward markedly at a concentration of about 0.008 mg/ml.

The results of the first two experiments allow us to make a test of two questions which are always prominent when dealing with labelled compounds; namely, (1) could the results obtained be due to a radioactive contaminant, such as the iodide ion ^{131}I or the labelled iodine molecule, and (2) has the iodinated RNase molecule been changed by the iodination procedure so that it no longer is taken up by the amoeba in the same manner as is the non-iodinated enzyme? To test these questions, seven solutions were prepared as indicated in Table I.

The solutions D, C, B and A all have the same total concentration of ri-

TABLE I. *Preparation of solutions for treatment of amoeba.*

These solutions correspond to the labelled points in Fig. 4.

Solution	Ml 0.2 mg/ml RNase (radioactive)	Ml 0.2 mg/ml untreated RNase	Ml phosphate buffer	Final concentration of RNase (mg/ml)
A	1	3	0	0.20
B	2	2	0	0.20
C	3	1	0	0.20
D	3	0	0	0.20
E	1	0	3	0.05
F	2	0	2	0.10
G	3	0	1	0.15

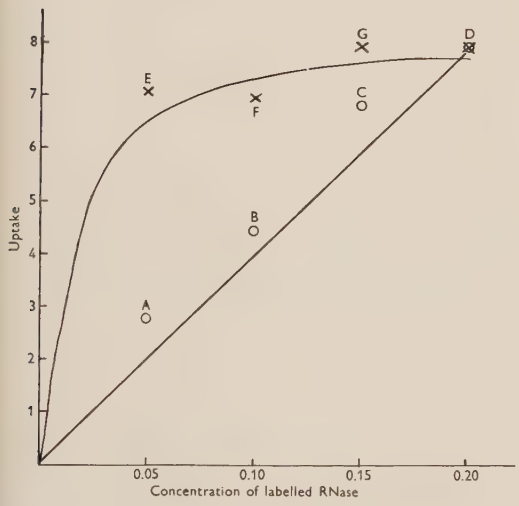

Fig. 4.

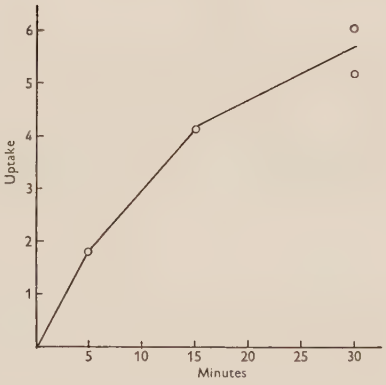

Fig. 5.

Fig. 4.—The uptake of labelled RNase by the amoeba as a function of the concentration of labelled RNase in the external medium. The solutions D, C, B and A all have the same total concentration of RNase but the concentration of the iodinated enzyme is progressively diluted from 100 to 25 per cent. The solutions E, F and G contain the same total concentrations of the iodinated RNase as do the solutions A, B and C respectively, but they are diluted with buffer instead of with enzyme solution. See Table I. The abscissa is the uptake of labelled RNase in units of 10^{-6} g/1000 amoebae, and the ordinate is the concentration of labelled RNase. The curve drawn through points E, F and G is the corresponding cross-plot of the data in Fig. 2 after 30 minutes of treatment. The straight line from the origin to point D is the "theoretical" line along which points A, B, and C should lie if the uptake of radioactivity from the mixture of labelled and non-labelled RNases is proportional to the ratio of the two species.

Fig. 5.—Uptake of RNase by the *Amoeba proteus* as measured by the enzymatic activity of the RNase extracted from the amoeba by dissolving the amoeba in NaOH and then extracting with H_2SO_4. The external concentration of the protein solution used to treat the amoeba was 0.1 mg/ml. The ordinate and abscissa are the same as used for Fig. 1.

bonuclease but the concentration of the iodinated enzyme is progressively diluted from 100 to 25 per cent. The solutions E, F and G contain the same total concentrations of the iodinated RNase as do the solutions A, B and C, respectively, but they are diluted with buffer instead of with non-labelled enzyme solution. As can be seen from Fig. 1, the concentrations of RNase

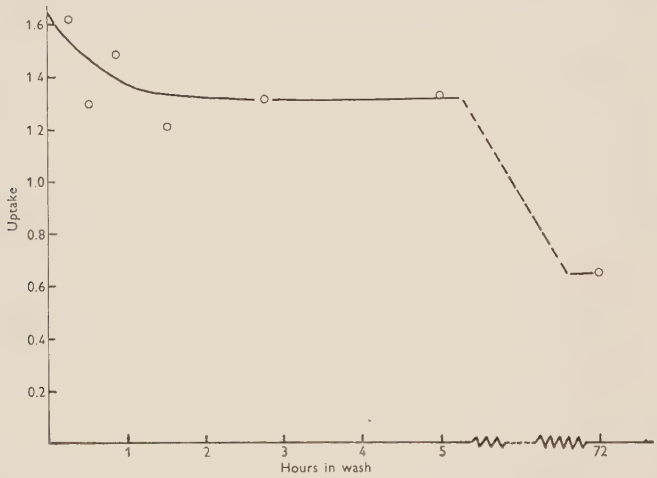

Fig. 6.—Release of RNase taken up by the amoeba as a function of time of washing in Chalkley's medium. The ordinate represents the quantity of RNase associated with the amoeba in units of 10^{-6} g RNase/1000 amoebae, and the abscissa is the time of washing the amoebae in minutes.

in all of these solutions are high enough to "saturate" the amoebae in 30 minutes. In Fig. 4 the uptake of radioactive RNase found after 30 minutes is plotted for each of these solutions. It can be seen that the amount of the labelled RNase taken up by the amoeba is almost the same for the solutions D, E, F and G. However the uptake of labelled RNase for the solutions C, B and A progressively decreases, clearly showing that the addition of the non-iodinated enzyme decreases the uptake of the radioactive compound. Had the measured uptake of the radioactivity been due to a contaminant, the points A, B and C should have coincided with the points E, F and G. Moreover it is seen that the non-labelled RNase competes favorably with the labelled RNase, indicating that the addition of iodine to the macromolecule has not radically altered those properties which influence the uptake of the enzyme by the amoeba.

A direct demonstration that the uptake of ribonuclease by the amoeba is a real phenomenon independent of the iodinating procedure can be performed by treating the amoeba with non-iodinated RNase and then extracting the

enzyme by chemical methods (see Materials and Methods) and measuring the quantity of enzyme removed by determination of the total enzymatic activity. This has been done, and the results are presented in Fig. 5.[1]

Two experiments were performed to follow the release by the amoeba of the enzyme which had previously been taken up from the solution. In the first of these experiments several hundred amoebae were treated for 15 minutes with a low concentration of the enzyme, washed thoroughly and left in a large quantity of Chalkley's medium. Periodically 50 amoebae were removed and washed and their content of RNase determined by measurement of radioactivity. The results of this experiment are presented in Fig. 6. In the second of these experiments, several hundred amoebae were treated for 15 minutes using a high concentration of RNase (0.1 mg/ml). The ribonuclease content of these amoebae was periodically determined. Some of the amoebae were placed in a solution of non-radioactive RNase prior to measurement of the radioactive RNase. The results of this experiment are presented in Fig. 7.

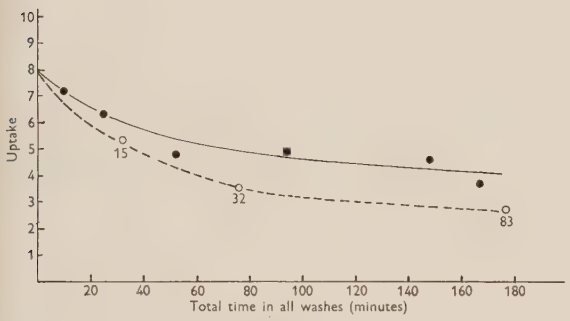

Fig. 7.

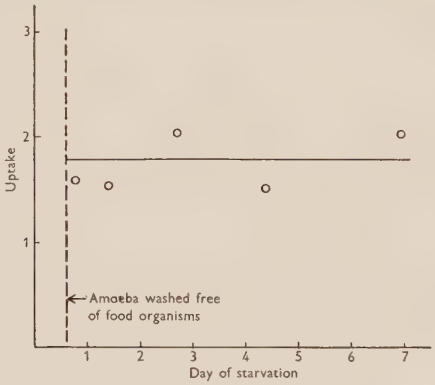

Fig. 8.

Fig. 7.—Release of RNase taken up by the amoeba as a function of time of washing in Chalkley's medium (●) or in a non-radioactive RNase solution (○). The abscissa represents the total time in all washes in minutes. The small numerals next to the unfilled points (○) indicate the length of time in the non-radioactive ribonuclease solution. The ordinate axis is the quantity of RNase associated with the amoeba in units of 10^{-6} g RNase/1000 amoeba.

Fig. 8.—Uptake of RNase (ordinate) after 15 minutes of treatment, as a function of the number of days of starvation (abscissa).

[1] Brachet *et al.*, *Biochem. J.* **66**, 14 P (1957) have measured RNase uptake by homogenizing treated amoebae and assaying directly the homogenate for enzymatic activity. They found considerably lower uptake values, indicating, perhaps, that much of the ribonuclease was still bound to cell debris.

These two experiments give us information concerning a rather important technical question. How sensitive is the measurement of the uptake of RNase by the amoeba to the length of the washing procedure? From Fig. 6 and 7 it can be inferred that the initial content drops at least 10 to 15 per cent in the first 15 minutes of washing. However, if the washing procedure is standard-

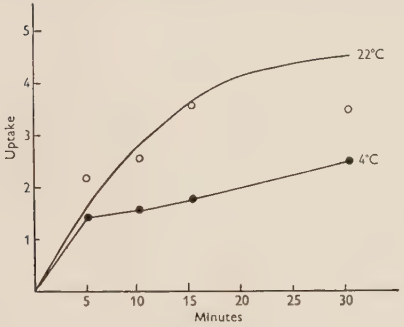

Fig. 9.—Uptake of RNases at 4°C (•) and at 22°C (○) from a solution of protein concentration of 0.02 mg/ml. The two experiments were performed simultaneously, but the curve drawn through the 22°C points has been taken for the corresponding concentration from Fig. 2. The abscissa and ordinate are the same as used for Fig. 1.

ized (as is the case for the experiments reported here—about 10 minutes), the error in reproducibility due to the release of the RNase during the washing process should be no more than 2 or 3 per cent.

An experiment was performed to determine the uptake of RNase by amoebae starved for various lengths of time. The amoebae were all taken from one stock preparation which had been washed free of food organisms the afternoon of day 0. They were treated for 15 minutes with a low concentration of radioactive ribonuclease, washed, and the quantity of the enzyme taken up by the amoebae determined. The results of this experiment are presented in Fig. 8. It can be seen that the uptake of the enzyme is not strongly dependent upon the length of starvation of the amoebae over a period of 6 days. For the experiments presented in this communication fresh amoeba preparations were used each week.

The influence of temperature upon the uptake of RNase has been studied by measuring the uptake of the enzyme at 4°C and at 22°C. In Fig. 9 the results of these experiments are presented. It can be seen that the uptake at the lower temperature is not inhibited during the first 5 minutes of treatment but that after 5 minutes a strong inhibition occurs and little additional protein is taken up.

The uptake of radioactive ribonuclease was studied in amoebae previously "saturated" with the non-labelled enzyme. The amoebae were first placed for 30 minutes in a solution of non-radioactive ribonuclease at a concentration of 0.1 mg/ml. This solution was then removed and immediately replaced

TABLE II. *Preparation of protein solutions of different pH for the treatment of amoebae as shown in Figure 11.*

Solution	Measured pH	Buffer	Ac or PO_4 molarity	Concentration of RNase
A	5.36	Acetate	0.003	0.05 mg/ml
B	6.10	Phosphate	,,	,,
C	6.95	,,	,,	,,
D	7.50	,,	,,	,,

by a solution of radioactive ribonuclease of the same concentration. The results of this experiment are presented in Fig. 10. It can be seen that the uptake of the labelled enzyme is very rapid during the first 5 minutes of treatment and then the rate changes markedly. After 30 minutes the uptake of the labelled enzyme is still 30 per cent lower than that found for amoebae with no pre-treatment.

The effects of pH upon the uptake of labelled RNase were studied with solutions prepared according to Table II. The amoebae were treated with

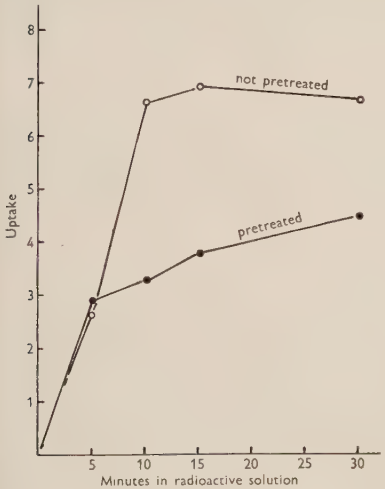

Fig. 10.

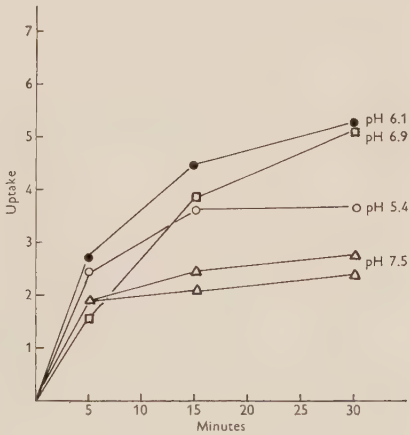

Fig. 11.

Fig. 10.—The uptake of radioactive RNase by amoeba previously "saturated" with non-labelled enzyme for a period of 30 minutes. The concentration of RNase pretreatment and treatment was 0.1 mg/ml. The curve labelled "not pretreated" was taken from the corresponding curve (0.1 mg/ml) in Fig. 2. The abscissa and ordinate are the same as used in Fig. 1.

Fig. 11.—The uptake of RNase by the amoeba as a function of pH. The solutions used were prepared according to Table II. The abscissa and ordinate are the same as used in Fig. 1.

these solutions for various lengths of time at room temperature (21 to 23°C) and the radioactivity of the labelled ribonuclease taken up by the amoebae measured in the Geiger counter. The results of these experiments are plotted in Fig. 11. It can be seen that after the first 5 minutes the uptake of the enzyme is less at the extremes of pH than it is for the intermediate values.

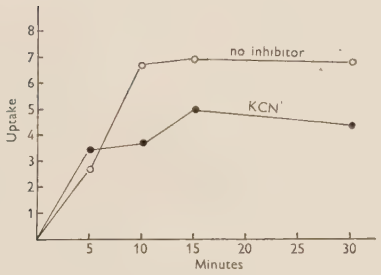

Fig. 12.

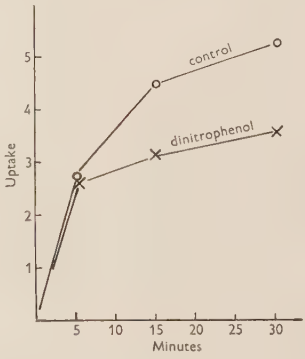

Fig. 13.

Fig. 12.—Uptake of RNase by the amoeba from a solution containing 10^{-3} *M* KCN and 0.1 mg/ml RNase (•). The amoebae were pretreated for 30 minutes in a 10^{-3} *M* KCN solution. The pH of these solutions was 6.2. A control experiment was not run simultaneously, but the curve (○) is the corresponding uptake curve taken directly from Fig. 2. The abscissa and ordinate are the same as used in Fig. 1.

Fig. 13.—Uptake of RNase by the amoebae from a solution containing 5×10^{-4} *M* dinitrophenol and 0.02 mg/ml RNase (×). The amoeba were pretreated for 2 hours with a 5×10^{-4} *M* dinitrophenol solution. The pH of these solutions was 6.12. A control experiment was not run simultaneously, but the curve (○) is the corresponding uptake curve taken directly from Fig. 11. The abscissa and ordinate are the same as used in Fig. 1.

The effects of inhibitors upon the uptake of RNase by amoebae was determined using 10^{-3} *M* KCN and 5×10^{-4} *M* dinitrophenol (DNP) at pH 6.2 and pH 6.12 respectively. The amoebae were first pretreated with the inhibitor for 60 minutes (KCN) or 120 minutes (DNP). The amoebae were then treated with labelled RNase in the presence of the inhibitor for various lengths of time. After treatment the amoebae were washed with distilled water by centrifugation and then by pipette and the uptake of radioactive RNase determined in the Geiger counter. The data are presented in Figs. 12 and 13. It is seen that during the first 5 minutes the uptake is not affected by the presence of the inhibitor, but that after this initial period the uptake is greatly reduced in comparison to that found for the non-treated amoeba.

The length of pre-treatment of the amoeba by KCN does not seem to influ-

ence the results obtained. With DNP, however, 2-hour pre-treatment with the inhibitor seems to be necessary if reproducible results are to be obtained.

Finally, if after treatment of the amoebae with RNase or cytochrome *c*, either in the presence or absence of KCN, the cells are washed *not with Chalkley's solution or distilled water but* with a 10^{-3} *M KCN solution*, as much as 50 to 80 per cent of the foreign protein can be removed from the amoeba in 20 minutes.

A few attempts to "see" the associated RNase by radioautography were made, but the high activity of the preparations made interpretation difficult. Amoebae were treated for 30 minutes in a solution of radioactive RNase at a concentration of 0.1 mg/ml. Treated and washed amoebae were frozen at liquid air temperature, dried in methanol, transferred to toluene, and finally embedded directly in paraffin. Sections were cut at 10 microns and deparaffinized in xylene. Strips of Ilford G-4 nuclear emulsion (poured and dried the previous day) were firmly pressed against the cut sections until the amoebae were intimately embedded in the emulsion. Both the amoebae and their radioautographical images remained together during exposure, development, and drying. The thickness of the nuclear emulsion and the large amount of radioactivity associated with the slices made resolution difficult, but the activity at the periphery of the amoeba was considerably greater than that found inside the amoeba.

DISCUSSION

A detailed examination of the data presented reveals several interesting points which must be taken into account in forming a hypothesis to explain the mechanism of protein uptake by the amoeba:

(1) At certain external concentrations of protein the amoebae are able to take up in 5 minutes as much protein as is found in fifty times their own volume of solution. This quantity of protein is sufficient to increase the dry weight of the amoeba by about 5 per cent.

(2) About one-half of the protein taken up by the amoebae is released into solution when the amoebae are washed for several hours, or is readily available for exchange when the amoebae are transferred to a labelled protein solution.

(3) A plot of the initial velocity of the uptake of protein is not a linear function of the external concentration of protein but instead it swings upward sharply as the external protein concentration exceeds a certain minimum level.

(4) At each of the various protein concentrations employed the uptake curve begins to level off and approach a plateau value which is characteristic of the concentration used, after 5 to 30 minutes of treatment.

(5) At the higher concentrations used the final plateau values are not much different and apparently a "saturation" point is reached at about the level of 8 γ RNase per 1000 amoebae.

(6) The observed initial rate of protein uptake is not greatly affected by KCN, dinitrophenol, low temperature and the extremes of pH.

(7) The subsequent rate of protein uptake after the first 5 minutes of treatment is strongly inhibited by KCN, dinitrophenol, low temperature, and the extremes of pH.

In an effort to explain these observations, the following hypothesis is advanced:

(1) The negatively charged membrane of the amoeba readily binds the basic proteins RNase and cytochrome *c*. This binding is reversible and an equilibrium point is rapidly reached; the quantity of protein bound to the membrane is a function of the external concentration.

(2) If the quantity of bound protein exceeds a certain minimum level, new sites capable of binding additional protein suddenly appear, perhaps due to the unfolding of pleated or globular structures. This initial increase in the number of binding sites is not sensitive to a lowering of the temperature, the extremes of pH, or the inhibitors employed.

(3) After the initial change in the structure of the membrane, the amoeba begins to ingest its own membrane, together with the protein bound to it. At the same time there occurs a synthesis of new membrane capable of binding more protein, and this synthesis is unfavorably affected by a lowering of the temperature, the extremes of pH, and the inhibitors employed.

The ingestion of membrane hypothesized in step 3 may well be the often described pinocytosis phenomenon, which appears to be induced by the presence of protein in the external solution [11]. Chapman-Andresen and Prescott [5], who have observed and studied the pinocytosis phenomenon microscopically, have described it as follows: "Examination under water immersion revealed immediate wrinkling or 'frilling' of the plasma membrane, followed within 30 to 60 seconds by the formation of numerous channels. The type of channel most frequently observed was a few microns in diameter and its length varied roughly between 15 and 50 microns; the longer channels were considerably twisted. Droplets were pinched off from the ends of channels as described by Mast and Doyle, sometimes whole channels disintegrated suddenly into droplets, but most frequently the whole

channel was pinched off close to its opening, thus forming a sausage shaped vacuole."

The proposed mechanism for the uptake of protein by the amoeba will now be examined in reference to the seven experimental observations listed above.

(1) The rapid uptake of a large quantity of protein by the amoeba may be explained by the proposed three-step mechanism. All three steps involve the binding of protein to the amoeba membrane. The proteins used in this study, RNase and cytochrome *c*, are both basic proteins and would have a net positive charge at pH 6 to pH 7. Therefore it would be expected that these proteins would bind to negatively charged regions on the surface of the amoeba membrane. This reaction involving the formation of a number of ionic or hydrogen bonds is probably rapid and limited only by the rate of arrival of the protein at the surface of the amoeba or by the rate of creation of new membrane. The quantity of protein taken up even in the early stages of the reaction, is many times more than is calculated by assuming that a mono-layer of RNase or cytochrome *c* is spread uniformly over a smooth sphere of volume equal to that of the amoeba (such a monolayer would contain about 0.25 γ of RNase per 1000 amoeba). The bulk of the bound protein must then be accommodated by binding sites located in multidinous surface irregularities.[1]

(2) An estimate of the quantity of protein bound to the external membrane after the amoebae have been in RNase solution of concentration of 0.1 mg/ml for 30 minutes can be made from the data presented in Fig. 10. If the membrane-bound protein is assumed to be equal to the protein readily available for exchange, the value can be obtained directly from the figure and is seen to be 2.8 γ of protein for 1000 amoebae. From the same figure, the total uptake of protein by the amoeba is seen to be 6.8 γ per 1000 amoebae. Therefore, 4 γ of protein must be taken up by ingestion of membrane. Assuming a density of membrane-bound protein of 2.8 γ per 1000 amoebae, the membrane would then have to be "turned over" by ingestion and resynthesis 1.4 times to account for the observed uptake of 4 γ of protein. If the average pinocytosis channel has a radius of 2 microns and a length of 30 microns (see Chapman-Andresen and Prescott [5] quoted above) it can be

[1] Little is known about the surface of the amoeba membrane, but electron micrographs indicate that it is composed of two layers; the internal layer is filbullar and the external layer is composed of fine globules [9]. If this is the case for the living amoeba as well as for the fixed and sectioned cell, it would not be surprising to find that the actual sub-microscopic surface area was many times greater than the macroscopic area. The membrane might then accommodate the large amount of foreign protein assumed to be bound to the surface of the cell.

calculated that 270 such channels would have to be formed and engulfed to account for this uptake of protein. At the same time the volume of solution taken up would be less than 6 per cent the original volume of the amoeba.

(3) According to the hypothesized mechanism for the uptake of protein by the amoeba, the initial rate of protein uptake will be dependent only upon the rate of binding of the protein to the original membrane. Since this uptake by binding is a reversible phenomenon, it will continue until an equilibrium state is attained. However, if the surface density of bound protein exceeds a certain minimum level, which from the data in Fig. 3 is at least 0.5 γ RNase per 1000 amoebae, a mechanism is triggered causing an increase in the number of binding sites on the membrane. It is these new sites which by binding additional protein directly cause the sharp swing upwards which is observed in the uptake curve in Fig. 3.

(4) At each of the various protein concentrations employed the plateau value of protein uptake finally obtained is characteristic of the concentration of protein in the external solution. This observation strongly suggests a reversible binding phenomenon; the plateau level representing the equilibrium concentration of the bound protein in the given solution. However, if an active transport mechanism is also involved by which the amoeba ingests a definite fraction of its own surface area once the triggering mechanism is activated, the total quantity of protein taken in with the ingested membrane would be a function of the concentration of bound protein per unit surface area. Since the concentration of bound protein is a function of the concentration of protein in solution, the total uptake would also be a function of the concentration of protein in solution.

In RNase and cytochrome *c* solutions of relatively high protein concentration (0.2 mg/ml), the uptake curves reach a plateau level after 5 to 10 minutes of treatment. At lower concentrations (0.02 mg/ml) the plateau region is not reached until roughly 30 minutes after the amoeba have been immersed in the protein-containing solution. From these observations it can be concluded that the rate of the active transport phenomenon is dependent upon the protein concentration; the total quantity of membrane ingested is taken in over a shorter period of time as the protein concentration is increased.

(5) In the solutions of high protein concentration the final plateau values are not much different and apparently a saturation point is reached at about the level of 8 γ RNase per 1000 amoeba. The hypothesized uptake mechanism would account for this observation if the following two conditions were fulfilled:

(*a*) At the higher protein concentrations the available binding sites are nearly saturated.

(*b*) As the protein concentration is increased, the amounts of membrane ingested and resynthesized attain relatively constant values.

(6) The observed initial velocity of protein uptake is not greatly affected by KCN, dinitrophenol, low temperature, and the extremes of pH. It is difficult to reconcile this observation with the third observation (the observed initial velocity of protein uptake is not linear but swings upward sharply as the external protein concentration exceeds a certain minimum level) by a two-step uptake mechanism. The hypothesized initial uptake is a simple binding of protein to the original membrane. The second stage in the uptake mechanism involves the "triggered" reaction which causes a sudden increase in protein uptake. This stage is not inhibitor, temperature, or pH sensitive, and it therefore must be different from the subsequent uptake mechanism which is sensitive to such treatments. Hence three steps are required to explain the uptake of protein by the amoeba.

The sudden rapid increase in uptake of protein by the amoeba as the protein concentration is gradually raised may be explained by the hypothesis that there appear additional protein-binding sites caused by the unfolding globular or pleated structures on the external surface of the membrane. This process may involve some increase in surface area and would then result in the "wrinkling or frilling of the plasma membrane" observed by Chapman-Andresen and Prescott [5] immediately after they had immersed amoebae in the protein-containing solution. The creation of additional binding sites is not inhibitor, temperature, or pH sensitive. Perhaps it is a response to changes in the electrostatic potential at the surface of the amoeba caused by the binding of charged protein molecules to charged groups on the membrane.

(7) The final part of the hypothesized uptake mechanism involves both the "taking in" of surface area—presumably by pinocytosis—and a resynthesis of the ingested membrane by an "active" mechanism (i.e. one sensitive to the effects of inhibitors, temperature changes, and pH). It is important to realize that the *ingestion of already bound protein does not directly result in a net increase in the uptake of protein by the amoeba.*[1] Additional new surface area must be created to replace the membrane which was absorbed, and it is

[1] This statement may be modified if large quantities of protein which would have been removed by the washing procedure are protected against washing by ingestion.

this new surface area which by binding additional protein directly causes the net protein uptake to increase.

Additional support for the hypothesized uptake mechanism comes from the work of Brandt who kindly allowed me to see his results prior to publication [3]. Using a fluorescent antibody to localize the foreign protein bound to fixed (freeze dried) and sectioned amoeba, this worker was able to demonstrate microscopically that large amounts of protein are bound to the surface of the amoeba membrane and also to the inside of the membrane surrounding "pinocytosis" droplets. Furthermore, he examined the pinocytosis process in the living cell and reported that the duration of pinocytosis was about 15 minutes.

It should be pointed out, however, that Brandt [3] was studying the giant amoeba *Chaos chaos*, while I have used the *Amoeba proteus* exclusively for the studies reported in this publication.

SUMMARY

The uptake of ribonuclease and cytochrome *c* from solution by living *Amoeba proteus* was studied using iodine 131-labelled protein. The uptake is slow until a certain minimum protein concentration is reached. The uptake then markedly increases. The uptake curve reaches a maximum within 30 minutes and then remains at a constant level. For external concentrations of 0.001, 0.01 and 0.1 mg/ml RNase the maximum uptake was 0.05, 1.9 and 6.8 γ RNase/1000 amoebae. Half of the protein, at a level of 8 γ/1000 amoebae, will come off the amoebae during a 3-hour treatment in distilled water. Inhibitors, the extremes of pH, and low temperature do not affect the observed uptake during the first few minutes of treatment, but after this short initial period the uptake decreases markedly or completely stops.

To explain these observations, a detailed hypothesis is advanced which involves both the binding of protein molecules to the external membrane of the amoeba and the subsequent ingestion of membrane by an active process presumed to be pinocytosis.

I would like to extend my appreciation for the many fruitful discussions with Dr. J. Brachet, Dr. L. Ledoux, and Dr. A. Ficq. I also wish to thank all the members of the Laboratoire de Morphologie Animale for helping to make my stay with them enjoyable and profitable.

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INTERFERENCE MICROSCOPE STUDIES OF CELL CONTACTS IN TISSUE CULTURE

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Received December 16, 1957

Fibroblasts *in vivo* or in tissue culture are usually arranged in an irregular meshwork, each cell having contact with its neighbours over only a few small areas of its surface. It is known that, in tissue culture at least, these contacts are adhesions [9, 6]. In an active culture they are constantly breaking and reforming [1]. It has been suggested that they play an important part in controlling the movement of fibroblasts [2] since it appears that when the movement of a fibroblast in a particular direction brings it into contact with another fibroblast, so that an adhesion forms, further movement in that direction is inhibited. This effect, so far only demonstrated in fibroblasts moving on a plane surface in liquid medium, was termed contact inhibition. Its existence was inferred from statistical data consisting partly of measurements of speed and direction of cells in relation to their contacts with each other; and partly of estimates of the frequency of overlapping of cells, since a consequence of the hypothesis of contact inhibition is that one cell should not be able to move across the surface of another. A similar statistical study of the relations betwen certain sarcoma cells and normal fibroblasts [3, 4] showed that contact inhibition did not occur between them, a mode of behaviour which it was suggested was related to the invasiveness of sarcoma cells. In order to follow up these observations, it is important to get a clear description of how these contacts between cells are made and of how sarcoma cells differ from normal fibroblasts in this respect. In our experience an interference microscope gives the best resolution obtainable of events at the margin of a cell. This is for two reasons. Firstly, the halo that surrounds cells in the phase contrast image is absent. Secondly, the contrast in the interference image indicates differences of thickness by differences of colour, producing a useful three-dimensional impression. What follows in this paper is a qualitative description of the movements and mutual relations of two kinds of fibroblast and of two kinds of sarcoma cell as seen in time-lapse colour films taken with an interference microscope.

METHODS

A Baker shearing type interference microscope was used, with a Zirconium arc of high intensity as a source of white light. Less frequently we used a compact source mercury arc, giving heterochromatic illumination, which seemed to provide better resolving power though with some sacrifice of colour contrast. The microscope was

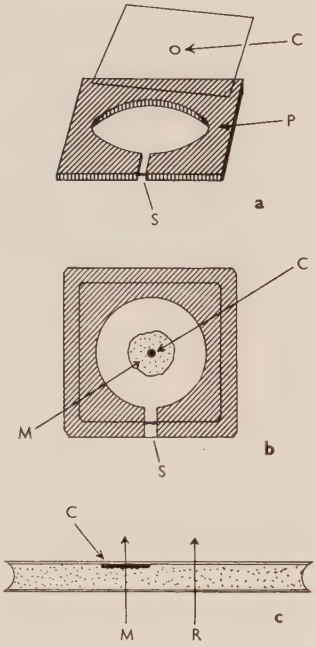

Fig. 1.—Method of mounting cultures for interference microscopy. *a*, "Dural" plate (*P*) with coverslip (*c*) sealed to lower surface. (*S*) opening for introduction of fluid. *b*, Culture (*C*) mounted with layer of fluid medium (*M*) between upper and lower coverslip. *c*, Arrangement of reference beam (*R*) and measuring beam (*M*) to give maximum colour contrast with the shearing type of Baker interference microscope.

fitted with a viewing attachment containing a mirror which acted as a partial reflector, so that the specimen could be kept under continuous observation during filming. A photocell could be introduced into the beam to determine the light intensity. A Cine-Kodak Special camera with Kodak time-lapse control was used for recording on 16 mm Kodachrome. Exposures were of 2 seconds' duration with the 40× objective which was used for most of our observations, and of 8 seconds' with the 10× objective, the interval between exposures being equal to the duration to exposure. The microscope was enclosed in a constant temperature box at 37°C.

Primary explants of heart from 7–10 day old chick embryos or of skeletal muscle from neonatal mice were placed in liquid hanging drop cultures. The medium was chick embryo extract, a drop of which was spread on a coverslip within a ring of clotted fowl plasma. Primary explants of mouse sarcoma 37 and sarcoma 180 (Crocker mouse sarcoma) provided the sarcoma cells. These explants produce very few cells when cultured on a glass surface. They were therefore placed in a drop of extract spread on the surface of a thin plasma clot itself placed on a coverslip. Immediately

before filming the coverslip was removed from its cavity slide, additional embryo extract was added to it, and it was inverted over a hole 15 mm in diameter in an aluminium sheet 2 mm thick (Fig. 1). The lower side of the hole was sealed with a second coverslip which just made contact with the hanging drop of fluid. In this way the two beams of light from the interference microscope condenser passed through a parallel-sided layer which was optically almost homogeneous. The position of the preparation was adjusted until the reference beam passed through a part of the field as clear as possible of cells.

Analysis of the film was chiefly made by dictating descriptions of events as the film was repeatedly projected. Frames were also printed on bromide paper at fixed intervals with the help of an analysing projector; the outlines of the cells could then be conveniently traced in ink and the prints subsequently bleached.

RESULTS

Fibroblast

Ruffled membranes.—When a fibroblast is moving on a plane surface its leading margin, as long as it is not in contact with other fibroblasts, consists of an exceedingly thin ruffled membrane closely applied to the substrate. Such a membrane may also occur, on a smaller scale, at other places around the periphery of a cell. The ruffles undergo continual small-scale changes in appearance, sufficiently fast to be detectable by direct observation, giving the cell here an irregular and continually changing border (Fig. 2). The degree of activity varies much from cell to cell. The ruffles occur mainly in the immediate vicinity of the membrane edge. Under the interference microscope they appear as a shifting pattern of thickened streaks and patches, which focusing shows are projections of the membrane surface away from the coverslip. The thickened streaks resemble folds or ripples in the membrane, sometimes 5–10 μ wide, and they usually seem to beat like highly irregular waves in a direction inward from the edge of the membrane. The inward movement of these undulations is much faster than the displacement of the whole cell. In mouse muscle fibroblasts the ruffles have a bigger amplitude of movement, occur over a relatively larger area of the membrane, and are less regular than in the chick heart fibroblasts. In none of these fibroblasts is the ruffling as big in amplitude or as disorderly as that seen in the membranes formed by macrophages.

Pinocytosis.—The membrane activity is at times visibly associated with pinocytosis, especially in the mouse muscle fibroblasts. Spherical droplets of the same colour as the medium appear just inside the free margin of the membrane amongst the ruffles and pass thence towards the nucleus, common-

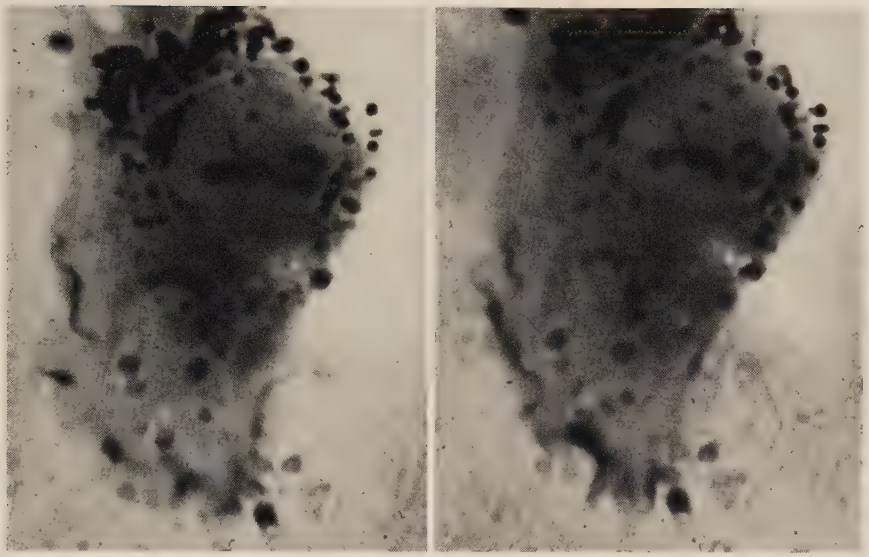

Fig. 2.—Chick heart fibroblast, showing ruffled membrane. The righthand picture was taken 10 seconds after the left. Photographed on Kodachrome with ×100 water immersion objective of the interference microscope.

only disappearing from view as they go. In a few perhaps pathological cells the droplets may be seen to accumulate at the base of the membrane where it thickens into the main body of the cell. Such a cell is shown in Fig. 3. Pinocytosis was never seen except in connection with ruffling, and when a membrane ceased ruffling pinocytosis stopped too.

Shape, size and position of membranes.—A large ruffled membrane is often narrower at its base than at its free edge so that it is approximately fan shaped. The lateral edges of such a membrane are usually less actively ruffled than the leading edge. A large membrane in a chick heart fibroblast may amount to $\frac{1}{3}$ or even $\frac{1}{2}$ of the area of coverslip occupied by the cell, and is responsible for the triangular form of the whole cell so often seen in chick heart fibroblasts. In mouse muscle fibroblasts membranes are narrower, though they may project further from the body of the cell; this may reflect not only the species and tissue difference but also the different age of donor [7]. These major membranes, in either type of fibroblast, usually merge gradually into the main mass of the cell with no sudden change of thickness (normal to the plane of the coverslip). There may be more than one large membrane on a cell, especially on a mouse muscle cell, though rarely more than two. One will generally be leading the cell, the other may project from any other place at

the cell's edge, even from the opposite pole. There is a continuous gradation in size of membranes from the major ones so far described through much smaller but still recognisably ruffled membranes, which often appear at the end of narrowed prolongations of the main body of the cell especially in mouse muscle, down to short tongues of very thin protoplasm momentarily projected from the cell and highly irregular in their ruffling. These smaller membranes may be projected from the cell body or from the sides of major processes of the cell body with a sharp transition in thickness and there may be many simultaneously present. It is likely, judging from the rapidity of their withdrawal that they are not attached to the glass substrate like the major membranes. There are also regions of the free lateral margin of the fibroblast, often quite extensive ones, which bear no membranes. This is particularly true of smooth margins of the cell body pulled into a tension curve through the combined effects of movement and adhesion. Regions of thin membrane devoid of ruffling may also appear (see below). These quiet parts of the margin occur both in stationary and moving cells.

Duration of a membrane.—A major ruffled membrane may last several hours though commonly altering in extent and shape and sometimes even splitting into two during this time. Smaller membranes can be very transient, some lasting no more than a few minutes. Membranes may be rapidly withdrawn into the cell in much the same way as they are formed, and this especially applies to the smaller ones. A larger membrane usually ceases to exist in rather a different way. It loses its ruffling, "freezing" so to speak, and retracting slightly towards the main body of the cell; but as it withdraws fine protoplasmic filaments show at its edge, obviously stuck to the glass. These become drawn out, distorting the margin of the cell into a series of scollops. Such strands may snap free from the substrate under the tension and retract, the whole membrane withdrawing as this happens, though it often uncovers further strands which in their turn become stretched and break. Fragments of cytoplasm seem occasionally to become detached in this way. On the other hand such a frozen membrane may stay for a long time extended, anchored by the strands and immobile. Sometimes only part of a large ruffled membrane may freeze in this way, while the rest continues its activity. Previous descriptions of the pseudopodia of fibroblasts on a plane surface as ending in fine filaments probably often refer to such immobile or retracting membranes. The filaments suggest that attachments of the membrane to the glass may be only at small localised areas. Very occasionally immobile membranes without visible anchoring filaments at their edge may be observed.

Relation of membrane to cell displacement.— A cell with a large and active

Fig. 3.

Fig. 4.

Fig. 3.—Pinocytosis in mouse muscle fibroblasts. Outline tracings, beginning at the top, taken from the film at intervals of 8 secs. Uptake of drops of medium by enclosure within the membrane can be seen in the cell on the righthand side.

Fig. 4.—The breaking of an intercellular adhesion. Outline tracings, beginning at the top, taken from the film at intervals of 8 secs. The establishment of tension during the first two frames leads to the sudden rupture of an intercellular adhesion.

ruffled membrane does not necessarily undergo bodily displacement in the direction of the membrane. It may be anchored by its other end to the glass surface or to another cell. It may even be pulled along in the opposite direction by an attachment to another cell itself in movement. Such attachments to the glass or to other cells do not, however, always limit movements: the region of attachment may become drawn out by the movement, distorting the whole cell body into a long process. As a rule where there is a major membrane in activity, the cell moves with this membrane in the lead; a less extensive membrane simultaneously present may fail to produce any apparent effect on

the direction of movement of the whole cell, but if such a secondary membrane enlarges relative to the initial leading membrane it may take over the leadership. In one cell an oscillation backwards and forwards was observed, each reversal of movement being associated with the rapid growth in size of a membrane at the trailing end of the cell and a shrinkage of the leading membrane. A particularly large membrane may not only lead the cell but may extend down one side and indeed there may be more of it on the side of the cell than anteriorly.

Formation of adhesions between fibroblasts.—When a ruffled membrane, large or small, leading or lateral, touches any part of the margin of another cell, either a static part or another ruffled membrane, it commonly becomes closely fused with it so that no line of demarcation can be seen. A few minutes after contact has been made the ruffling movements of the membrane stop. There were a few instances of the continuation of some ruffling, usually but not always associated with the presence of a nearby free edge of the membrane which was continuing its movement. Sometimes after contact has been made the fused membrane seems to retract a little, judging by the tension curves which appear on the free sides of the membrane, where all undulation ceases. But this retraction does not always happen, though it is very unusual for a membrane which was extending at the time of contact to continue to do so. Occasionally the membrane may extend at one of the free sides, being thereby diverted along the edge of the cell with which contact has been made. Since the free membranes are often broad, they may meet another cell on a wide front, and the initial adhesion may be established at several points close together. Adhesion of the whole breadth of the membrane may follow. Most newly-formed contacts are changing all the time. The membrane which has made the contact may retract slightly revealing numerous protoplasmic strands attached to the cell, of a similar calibre to those which as already described may appear attached to the coverslip. Having slightly retracted, the membrane may rejoin closely or the extent of its contact may shift. These changes occur in the absence of any sign of tension in the cytoplasm making the contact. In relatively closely packed cells each of which has established adhesions with several other cells and is undergoing little displacement, adhesion may however last apparently unchanged for many hours. Some of these adhesions distort the cells, pulling them out into long and relatively thick connecting processes.

If the membrane establishing an adhesion was a leading one, the displacement of the cell in the original direction usually ceases. Another membrane, or sometimes the lateral part of the same membrane, takes up the leadership

of the cell. Consequently the cells tend to remain in a monolayer, as previously described [2].

Loss of adhesion between cells.—Adhesions may be as it were spontaneously withdrawn during the various rearrangements of the area of contact which commonly occur. In general no movement of the contacting cells in opposite directions is involved in such a breakdown of an adhesion. The change is analogous with the withdrawal of a free membrane from the glass surface which we have previously mentioned. Adhesions may, however, also be pulled apart by divergent movement of the two cells, which tends to occur through the assumption of leadership by a ruffled membrane other than the one that formed the adhesion. Fine strands may link the cells and become pulled out, often distorting both cells considerably (see Fig. 4). The break when it comes is sudden and the two cells then snap apart, occasionally in several jerks as successive connecting strands break. When or even before all connecting filaments have broken free, the membrane which had formed the adhesion may resume its ruffling, though it may also withdraw completely into the cell body.

Failure of adhesion formation. –A ruffled membrane, when it meets another cell, particularly an elongated process of the cell body, may sometimes overshoot so that the tip of the membrane appears on the other side of the cell process, though substantially in the same plane of focus. An adhesion may nevertheless be formed by a more proximal part of the membrane and the whole membrane may then withdraw and lose its ruffles. This overshooting seems to be more common in the mouse muscle than in the chick heart fibroblasts. The former are more straggling in form, and one mouse muscle fibroblast may cross entirely over a process of another. Two instances were observed, one amongst mouse muscle, the other amongst chick heart fibroblasts, of cells which persistently failed to form adhesions. A mouse muscle cell, for instance, otherwise normal in appearance and preceded by an undulating membrane crossed over the surface of two other fibroblasts and partially crossed a third, though it then succeeded in forming an adhesion with this last cell. The cells that it crossed were adhering normally to other cells.

Movement of adherent cells.—Not all fibroblasts in movement are preceded by a ruffled membrane. When a cell is adherent to others all around, a certain amount of minor oscillation may occur. Cases are observed where a ruffled membrane leading a cell has made a contact with another cell, and the ruffling has stopped, but the cell body continues its movement for a short time, approximating the two cells. Movement without a discernible leading membrane may also be seen in closely packed sheets of chick heart fibroblasts moving

in a common direction, usually radially away from the explant. These cells are usually elongated in the direction of movement, and they move consistently except that occasionally they may reverse their direction. Lateral movement in relation to this polarisation is rare. Part or all of the anterior end of such a cell may be at any time free from contact with other cells and may have a ruffled membrane. But the entire front end of a steadily moving cell may be adherent to the hind end of the cell in front by a thin membrane of even thickness, devoid of ruffling and in fact similar to any other membrane which has established an adhesion. In such closely packed cells there may be a good deal of activity at the cell margin in the narrow space between parallel cells, usually consisting of small and quickly changing membranes and projections put out for varying times and then retracted. Many of these form temporary links with the neighbouring cells. The hind end of such a cell is sometimes entirely devoid of projections and appears simply as a rounded bulb. The whole sheet of cells slides steadily over the coverslip with only slow changes of arrangement amongst its constituent cells.

Sarcoma Cells

Appearance of individual cells.—This description is confined to the rounded form of sarcoma cell of S37 and S180, which has been the main subject of previous analysis of contact inhibition [4]. These cells are much thicker than fibroblasts and are of compact form, often nearly circular in outline. Round the visible periphery numbers of very fine rather spiky processes are present in very active movement. In the circular cells these are randomly distributed all round, but in cells which have departed a little from the circular form the fine processes may be more localised, often being more pronounced at the narrowed leading end of the more elongated cells. Besides these very fine processes, which are so active that their changes can easily be detected by direct observation, there are sometimes more substantial projections of the cell body which change less rapidly in form. When the cell is displacing itself, especially when moving fast, its whole shape may change and in particular it may become somewhat elongated into an oval or pear shape oriented in the direction of movement. Such changes of shape are only temporary. A moving cell, however, does not necessarily alter its shape in this way; it may simply glide along maintaining its circular form. Occasionally the distortions of a cell may elongate it at right angles to its direction of movement. In spite of the intense activity of the processes at the surface of these cells displacement is slow and irregular. It may be added that these rounded sarcoma cells are readily distinguishable from macrophages which also occur

in sarcoma cultures. The macrophages are smaller, much more changeable in form and they have extensive veil-like undulating membranes quite different from the surface projections of the sarcoma cell.

Mutual behaviour.—When they are densely packed the rounded sarcoma cells move over each other's surfaces without apparently much mutual influence. No information of adhesions between contacting cells, as shown by the drawing out of processes between them as they move apart or by their jerking away from each other when they separate, was detected, apart from one instance of a cytoplasmic connection between two separating cells. As far as can be judged the superficial activity continues unaltered in the immediate neighbourhood of contact between two cells. The film gives the impression however that to some extent sarcoma cells tend to stay clumped once they have made contact with each other; though there is no lack of instances in which a cell moves away from a clump.

Interaction of sarcoma cells and fibroblasts.—Sarcoma cells can be observed moving freely over all exposed parts of the surface of a fibroblast. They may pass over a ruffled membrane of a fibroblast momentarily obscuring part of it but as far as can be judged without altering it in any way; their own movement proceeds unhesitatingly and the activity of their own surfaces seems almost unaltered. It appears however that there is a tendency for a sarcoma cell when it makes contact with a fibroblast to become distorted in shape, extending a little over the surface of the fibroblast with a localised exaggeration of the surface processes at the tip of the projection. This does not always happen; and similar distortions may occur apart from contact with fibroblasts, but less frequently. No sign can be detected of any specially firm or long-lasting adhesion between sarcoma cell and fibroblast. The latter is merely a substratum for the former. A sarcoma cell may move off a fibroblast as rapidly as it moved on. It may sometimes cease its locomotion when it is on the surface of a fibroblast, as it does on another surface, and it is then carried along by the movement of the fibroblast which proceeds unaltered. In such cases it can be seen that the surface of the fibroblast to which the sarcoma cell is temporarily attached is moving at the speed of the whole cell.

The film gives a very clear impression that sarcoma cell and fibroblast do not react similarly to the same local stimuli. Fibroblasts may be seen proceeding in one direction along the surface of the clot on which the cultures were made, while sarcoma cells in the same field are proceeding less regularly but predominantly in the opposite direction, both sets of cells travelling away from their own explant.

DISCUSSION

The present paper provides a description of contact formation between fibroblasts, which is required before an anlysis of the phenomenon of contact inhibition [2] can begin. A fibroblast moving on a plane surface, if its forward end is free of other fibroblasts, seems always to be preceded by a pseudopodium in the form of a ruffled membrane. Consequently it is such a membrane which makes contact with other fibroblasts. The contact when established is a close adhesion, as many previous workers, e.g. Lewis [9] and Kredel [6], have reported. It may be broken if the cells move in different directions, but the strength of the adhesion is manifested by distortion followed by sudden retraction of the adhering cytoplasm and a jerking apart of the cells [1]. In addition to adhesion, a cell whose membrane makes a contact usually suffers three other consequences. First, the ruffling activity of the membrane stops. Second, the membrane, if it was expanding, ceases to expand and may contract. Third, the displacement of the whole cell in the direction of the membrane is checked (contact inhibition), though not necessarily immediately (Abercrombie and Heaysman [1] in fact found a drawing together of cells immediately after contact had been made). These three changes occasionally occur, in association, when the leading membrane has made no contact with another cell.

The ruffling activity can be seen to be concerned with pinocytosis. In this connection the occurrence of neutral red staining vacuoles at the base of leading pseudopodia and the positive correlation of their number with the locomotory activity of the cell, described by Carrel and Ebeling [5], suggests the hypothesis that these vacuoles are at least partly the result of pinocytosis. It seems improbable that the ruffles are concerned in doing the work of locomotion. The direction of displacement of the whole cell is not a resultant of the direction of all its ruffling activity. The ruffles move in a direction essentially normal to the periphery of a membrane, wherever it is, so that most of the ruffling movement may be very asymmetrical in relation to the direction of cell movement. Furthermore displacement may occur without a ruffled membrane, or with a very restricted one, in closely packed fibroblasts moving together.

It is clear however that there is an association between a ruffled membrane and the locomotion of the whole cell, because if the front end of a cell is free of other cells, it seems always to consist of such a membrane (though other, less extensive, membranes may be simultaneously present at other parts of the cell's edge); moreover cessation of ruffling in a leading membrane, either

through contact with another cell (contact inhibition), or more rarely "spontaneously", stops cell locomotion in that direction. As a working hypothesis it may be suggested that a ruffled membrane tends to control the direction of whatever mechanism it is that moves the whole cell; and that when a cell bears more than one ruffled membrane, these may compete for control of direction as Weiss and Garber [10] proposed for fibroblast pseudopia when the cells are moving in a clot. The competition may be for cytoplasmic flow from the main mass of the cell, but the mechanism of fibroblast movement is so uncertain that further discussion of this aspect is not profitable.

Contact inhibition seems then to consist of the abolition or reduction of the power of the leading membrane to direct the general cell movement, and hence the assumption of dominance by another membrane, newly arising or previously at a competitive disadvantage, which then redirects the cell. Redirection may also occur in a similar way in the absence of contact inhibition, possibly through slight advantages conferred on membranes by differences of the substratum. For fibroblasts, as well as adhering to each other, adhere to the glass or other substratum on which they are moving; though usually this adhesion must be in some way different from that to other fibroblasts, otherwise the cell would be able to move over other fibroblasts as it can over glass.

It seems probable that the locomotory mechanism of a fibroblast does not require to be led by an undulating membrane. Close-packed fibroblasts in culture may move together in mutual contact, and some of these cells have no ruffled membrane in the lead, being apparently attached to the cell or cells in front by a membrane devoid of ruffling. The formation of an adhesion does not therefore stop movement in the original direction provided the cell to which the adhesion occurs is itself moving in that direction; and, perhaps one should add, provided new ruffled membranes have no space to develop extensively from other parts of the cell edge before they are inhibited by contact with neighbours. It may be of course that these cells that move without a ruffled membrane are being passively dragged along. Possibly, however, the locomotory mechanism is orientated by tension on the cell, and both a ruffled membrane and attachment to a moving cell provides such a tension. The phenomenon requires further analysis, particularly because of the similarity of fibroblasts in such a situation to the cells of an epithelium [8].

The sarcoma cells on which observations have so far been made were of the more rounded kind which in culture are most actively moving and most actively invasive of a contiguous colony of fibroblasts. It is probably a mechanical consequence of the shape of these cells that they move over the surface

of the flattened fibroblasts and never vice versa. The style of movement of the sarcoma cells is very different from that of fibroblasts. They have intense and continuous activity over their whole visible surface. There is little corresponding to a leading ruffled membrane, though the activity at their surface seems often to be of greater amplitude at the leading end, and to take place on a major projection of the cell which might be called an incipient pseudopodium. Nothing like the contact phenomena observed between two fibroblasts occurs when a sarcoma cell meets another sarcoma cell or a fibroblast. There is no cessation of the sarcoma cell's surface activity corresponding to the cessation of ruffling, and, at least when a sarcoma cell meets a fibroblast, its movement is not checked. This is in agreement with the immunity of these sarcoma cells to contact inhibition by fibroblasts [4]. The sarcoma cell must adhere in some degree to a fibroblast, or it would not be able to move over its surface; and the fibroblast does not appear to be exactly equivalent to any other substratum, since these sarcoma cells gave the impression of becoming a little distorted in general shape when on a fibroblast's surface. But the distinction that a fibroblast makes between another fibroblast and a glass or fibrin substratum is not made by a sarcoma cell; both are available for the latter's movement.

SUMMARY

1. The movement and interrelations in tissue culture of chick heart and mouse muscle fibroblasts, and of the rounded cells of mouse sarcoma 37 and sarcoma 180, have been described from time-lapse colour films taken with an interference microscope.

2. A fibroblast moving on a plane surface with its leading pole free is preceded by a ruffled membrane. Other, smaller, ruffled membranes may occur elsewhere around the periphery of such a cell. The large ruffled membranes are associated with pinocytosis.

3. When a ruffled membrane of one fibroblast touches any part of the peripheral surface of another fibroblast, it usually forms a close adhesion to it. The ruffling then stops, the membrane if growing in size ceases to expand and the whole cell if moving in the direction of the membrane soon ceases to do so. A lateral membrane may then lead the cell in a new direction. Adhesions are usually temporary. The adhesion and cessation of activity of a leading ruffled membrane appear to be the visible expression of the contact inhibition previously reported. A somewhat similar cessation of activity may occur in a ruffled membrane leading a cell, in the absence of contact with other cells,

producing a change of direction when another membrane takes over the leadership.

4. A rounded sarcoma cell has a very actively moving surface, but not in the form of a ruffled membrane. Little or no adhesion or change in surface activity, or in locomotion, can be seen in a sarcoma cell when it meets another, or meets a fibroblast, or conversely in a fibroblast when it meets a sarcoma cell.

We should like to thank Professor A. Haddow for his interest in this work. We should also like to express our grateful thanks to the following colleagues who assisted us in various ways: Mrs. M. Blazey, Dr. D. M. Easty, Dr. J. E. M. Heaysman, Miss H. M. Karthauser, Mr. K. G. Moreman and Mr. F. E. Speed. One of us (M. A.) is indebted to the Nuffield Foundation for financial help.

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THE TIME SEQUENCE OF EARLY EVENTS IN THE FERTILIZATION OF SEA URCHIN EGGS[1]

II. THE PRODUCTION OF ACID

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Received December 17, 1957

Within about five minutes after fertilization, manometric methods are capable of detecting a burst of carbon dioxide release from the sea water surrounding sea urchin eggs. The cause of this CO_2 release is the production of an as yet unidentified "acid of fertilization" [8, 9, 10, 11]. Since there is a definite lag in the detection of this acid by manometric means, the exact time-course of acid production is not known. If the acid were released soon after sperm penetration (during the latent period; cf. [2]), it might function as a kind of rapid block against polyspermy. Such a suggestion was made by one of us [5] in the light of evidence that acid can inhibit fertilization [14]. If acid production were delayed until the cortical reaction, a polyspermy-preventing role could be excluded, as the egg would need some other mechanism to prevent penetration of supernumerary sperm during the first 15–20 seconds after attachment of the first successful spermatozoon [2]. Acid production during the cortical reaction might, however, mean that the acid had come with or from the breakdown of the cortical granules or had been otherwise released by the cortical change. On the other hand, delay in the production of the acid until after the cortical reaction would render the latter improbable, unless the acid and fertilization membrane were of such a character that egress of the acid was very slow.

It seemed worth while to investigate some of these possibilities with a method more sensitive to the time-course of acid production than manometry. In the present work, the rapid fall in pH following nearly synchronous fertilization of a dense suspension of sea urchin eggs in unbuffered (bicarbonate-free) sea water [1] was measured with a Beckman Model H pH meter.

Eggs of the sea urchins *Psammechinus miliaris* from the Swedish west coast, and *Arbacia punctulata* from Woods Hole, Massachusetts, were used in this

[1] This investigation has been supported by a research grant (C-3022 (C)) from the National Cancer Institute, National Institute of Health, Public Health Service, Bethesda, Md., U.S.A.

study. Eggs were rendered jelly-free by brief treatment with sea water at a pH of 5.8 [4], and then washed several times in bicarbonate-free artificial sea water made from purest grade salts and pyrex-distilled water. One *Arbacia* female at the height of the reproductive season produced enough eggs for the experiment shown in Fig. 1; however, it was necessary to pool the eggs of several *Psammechinus* females to equal the egg density and degree

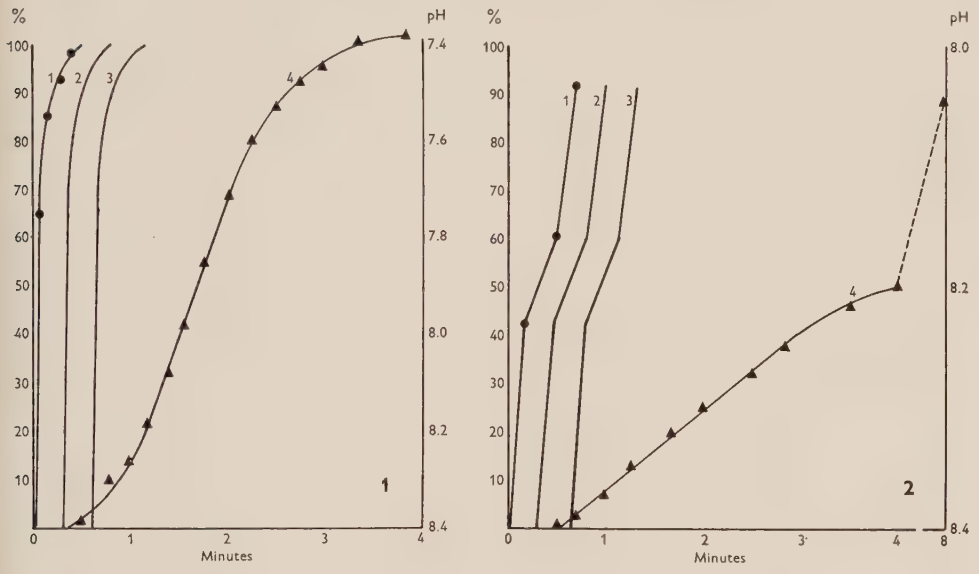

Fig. 1.—Time-course of acid production in *Arbacia eggs*. See text.
Fig. 2.—Time-course of acid production in *Psammechinus* eggs. See text.

of acid production found in *Arbacia* (Fig. 2). One hundred milliliters of a dense suspension of eggs in unbuffered sea water (pH 8.4) were inseminated with 10^7 sperm per ml suspended in the same medium at 20°C. Changes in pH were followed on the pH meter at intervals of about 15 seconds; mixing was accomplished by slow rotation of a glass stirrer. At intervals of a few seconds, aliquots of eggs were withdrawn for treatment with 0.001 per cent sodium lauryl sulfate to determine fertilization rate according to the method of the Hagströms [6] (curve 1 in both figures). Control suspensions of unfertilized eggs or of sperm affected the pH of the medium only slightly over an extended period.

Figs. 1 and 2 show the results obtained on parallel experiments with gametes of *Arbacia* and *Psammechinus* respectively. Fertilization rate curves

(curves 1 on both figures) were based on counts of 200 eggs. The middle curves represent the approximate beginning and end of the latent period and cortical reaction from the data of Allen and Griffin [2]. Curve 4 on both figures shows alterations in pH of the medium. The difference in shape of the fertilization rate curves in the two graphs is probably attributable to the pooling of eggs in the *Psammechinus* experiment. It should be noted that acid production is more gradual and continues longer in *Psammechinus* than in *Arbacia*.

It should be pointed out that these experiments are subject to some error. It is conceivable that some buffering action might be introduced by the addition of jelly-free eggs to the bicarbonate-free sea water. This might come about either by the production of CO_2 by the eggs during the course of the experiment, or by the buffering action of proteins near their isoelectric point at the egg surface. The buffering action of CO_2 is probably unimportant because of the previously mentioned observation that unfertilized eggs alone did not significantly alter the pH of the medium over the time period involved in the experiments. The egg surfaces are unlikely to contribute much buffer capacity to the system since sea urchin eggs (with or without jelly) exhibit a high negative zeta potential, indicating a highly acidic character of their surfaces [3]. The curves for the production of acid (Figs. 1 and 2) may thus include some delay; that caused by buffering action of the eggs would seem to be minor. There is also some delay involved in the detection of the acid after it has emerged from the egg. However, this would be less than the delay in measuring the fall in pH when a drop of dilute acid was added to a beaker of eggs in which final pH was reached in 1–2 seconds.

This method does not give information on the exact time of the *intracellular* production of acid, but rather an approximate estimate of the time that this acid arrives *outside the egg*, where presumably it would have to act to influence the fertilization process. For this reason, it is clear that acid production from the egg as a whole occurs too late to play any role in the prevention of polyspermy. It is also clear that the acid is not ejected all at once during the breakdown of the cortical granules. However, it does look as though the acid begins to appear in the medium during the visible cortical changes. The acid of fertilization reflects metabolic changes brought about by the activation process [13]. It should be remembered that (1) artificially activated eggs produce acid [11], and (2) homogenates of unfertilized eggs prepared with calcium-free sea water produce acid on the addition of calcium [7, 12]. In the latter case, the cell cortices would certainly have been destroyed as functional units, but certain subunits may remain stable in absence of calcium.

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THE MODE OF CHROMOSOME DUPLICATION IN *CREPIS CAPILLARIS*[1]

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Received December 17, 1957

By the use of tritium-labeled thymidine which is a selective label for DNA (deoxyribonucleic acid), chromosomes were shown to conserve their original DNA during duplication and to distribute it equally to the two daughter chromosomes [5]. These labeled daughters, when they duplicate in the absence of labeled precursors, yield one labeled chromosome and one free of label. The interpretation of these results requires the chromosome to be two-stranded before duplication. The two strands separate and each has a new strand built along it which will be labeled when thymidine-H^3 is supplied to the cell during the duplication. All of the chromosomes appear labeled at the succeeding division, but they reveal that each carries an unlabeled strand when they duplicate a second time. This mode of duplication was first demonstrated in the cells of the roots of *Vicia faba*, the broad bean. Since then the same mode of duplication was shown to occur in the lilaceous plant, *Bellevalia romana* [4]. In addition evidence from sister chromatid exchanges indicated that the two strands of the chromosomal duplex are different in some structural sense.

All of this evidence would strongly favor the view that these two species are typical in that all chromosomes are two-stranded and duplicate in the same way. However, Plaut and Mazia [1] obtained evidence that *Crepis capillaris* differed in that the distribution of labeled DNA at the first division following incorporation of thymidine-^{14}C was often very unequal. Since autoradiographs with ^{14}C do not give good enough resolution to follow the labeling in individual chromosomes, they estimated grains over the two daughter nuclei at anaphase or telophase. Some of these appeared to have two times as much radioactivity as the sister nucleus. However, the distribution of label at the second division after labeling was not determined.

[1] The investigation has been supported in part by grants from the U.S. Atomic Energy Commission, Contract AT (30-1)-1304 and from the Higgins Fund of Columbia University.

By the use of tritium-labeled thymidine the chromosomes of *Crepis* were examined for the two-stranded property. In addition evidence was obtained that chromosome duplication begins at the ends and proceeds toward the centromere.

MATERIALS AND METHODS

Seeds of *Crepis capillaris* were germinated on moist filter paper at 25°C and grown for various times with the roots immersed in films of mineral solution. The solution used for labeling contained 2 3 μg of thymidine-H^3 (300–400 μc/μ mole). Roots were fixed after 5, 6, 10 and 18 hours in the isotope solution. When cells were required that had gone through one duplication with labeled precursors and one duplication without labeled precursors, the roots were washed after 6 hours and placed in mineral solution free of labeled thymidine. To increase the frequency of metaphases, colchicine was usually added to the mineral solution after the roots were taken from the thymidine solution.

Roots were fixed in acetic acid–alcohol (1:3) for several hours, hydrolyzed for 6 minutes in 1 *N* HCl at 60°C and stained by the Feulgen reaction. The roots were squashed on slides in 45 per cent aqueous acetic acid. The preparations were frozen on dry ice, the cover glasses removed and the slides immersed in acetic–alcohol for a few minutes and then in 70 per cent ethanol. The slides were finally rinsed in water and stripping film applied as described elsewhere [2].

RESULTS

Only the nucleus is labeled when cells are grown in tritium-labeled thymidine (Figs. 1*a* and 1*b*). When the chromosomes condense at the prophase prior to a division, the label is seen to be restricted to the chromosomes (Fig. 6*a*). Although *Crepis* has only 6 chromosomes in diploid cells (Fig. 2) they are small enough for resolution of the two chromatids in each chromosome of the complement to be difficult even with the high resolution possible with tritium. The chromatids must be separated well as shown in Fig. 2. This condition is obtained in some of the cells after 6–10 hours growth in a colchicine solution. For convenience in referring to the different chromosomes, the designations shown in Fig. 2 will be used. Chromosome I is the largest one, chromosome II the medium sized one and chromosome III the smallest one.

Labeled anaphases begin to appear after the cells have been growing in the thymidine-H^3 solutions for 5 -6 hours. By 10 hours all cells that reach anaphase are labeled. Flattened anaphases (Fig. 3) show that both daughter chromosomes of each morphological type are similarly labeled. Tetraploid *c*-metaphases, produced by placing the roots in colchicine solution free of

labeled thymidine after 6 hours growth in the labeled solution, show chromosomes with one chromatid labeled and one unlabeled (Figs. 4*a* and 4*b*). Such cells to become tetraploid must have gone through one chromosome duplication while in the colchicine, and therefore, since they were labeled. Because it was rarely possible to analyze all of the chromosomes in tetraploid cells due to overlapping or failure of all chromatids to separate and flatten well, appropriate timing also was employed to obtain diploid cells at the second division after labeling occurred. When cells are grown for 6 hours in thymidine-H^3, then 4–6 hours in solution free of label and finally in cholchicine for 6 hours, most of the labeled cells showed the distribution of label shown in Fig. 5. Here the metaphase has been blocked with colchicine so long that the chromatids have finally completely separated. However, by matching them on the basis of their morphology and position, it is clear that each chromosome has produced one labeled and one unlabeled chromatid (daughter chromosome). No chromatid exchanges occur in this cell, but they occur with a rather high frequency in many of the cells; note the one in Fig. 4*b*.

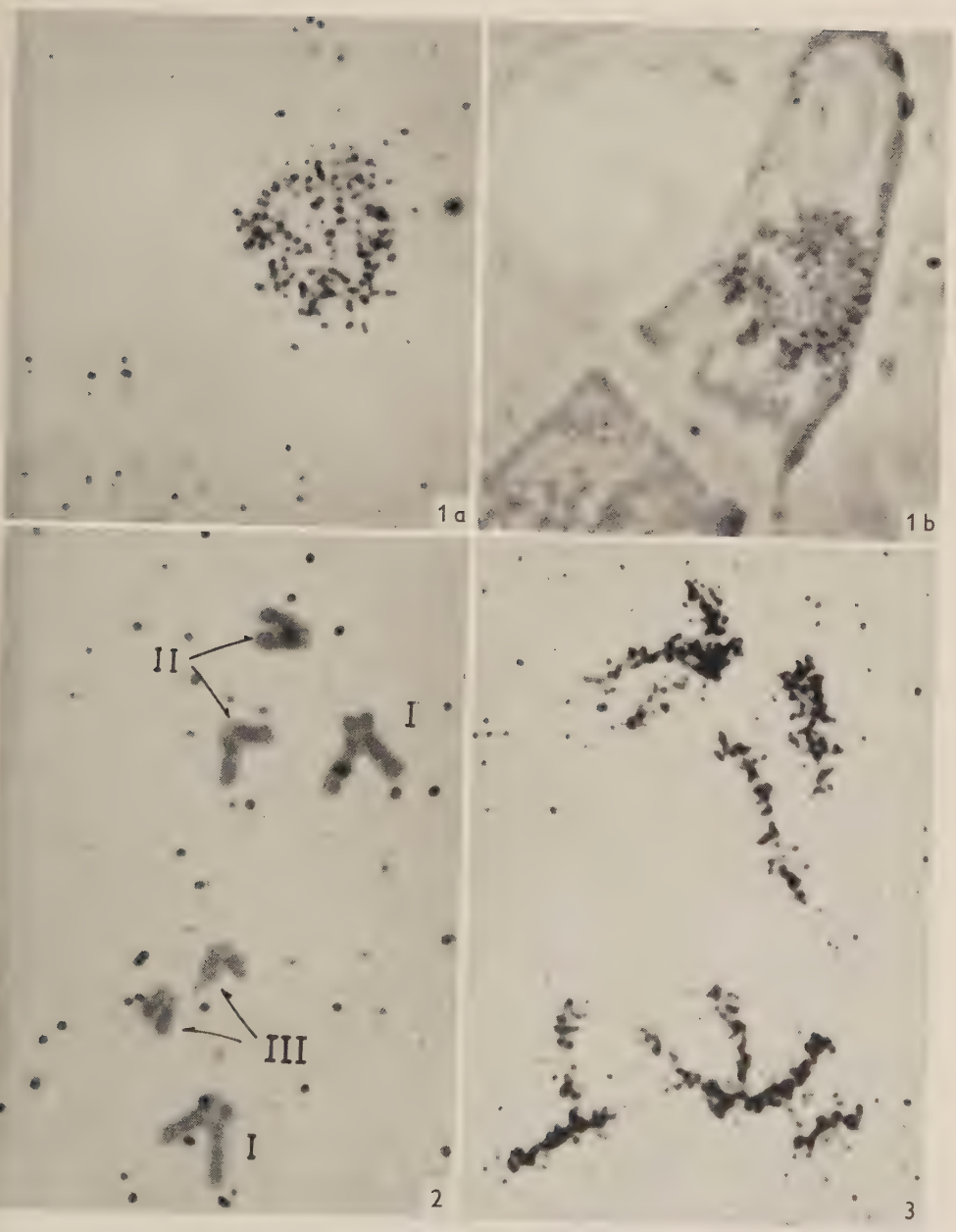
When cells were grown for 6 hours in thymidine-H^3, 10 hours in solution free of label and finally in colchicine for 8 hours all of the cells at metaphase were diploid and from one to six of the chromosomes were labeled. These were evidently almost exclusively at the third metaphase after labeling occurred.

An examination of the first chromosomes to appear labeled after growth in thymidine-H^3 solution (cells fixed after 5–6 hours) revealed an interesting feature of the distribution of label. Some of the prophases showed a gradient of labeling along the chromosome (Fig. 6*a* and 6*b*). In addition anaphases and metaphases in some instances showed that the label occurred only in the vacinity of the centromeres (Fig. 7*a* and 7*b*). After 10 hours in the thymidine-H^3 chromosomes regularly are labeled uniformly along the whole length in so far as resolution can reveal. In chromosome I, which has two arms that are separately resolved in prophase, the two arms are usually differently labeled, for example the chromosome I in the upper right of Fig. 6*a*.

Fig. 1.—Cell of the root tip of *Crepis capillaris* which had grown in thymidine-H^3 for 6 hours. (*a*) Photographs of silver grains in the emulsion; (*b*) phase photograph of the cell below the same area of the film. × 2500.

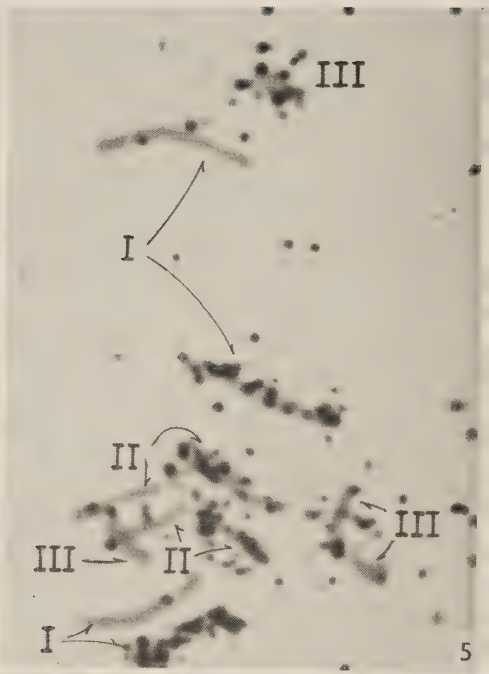
Fig. 2.—The diploid chromosome complement of *Crepis capillaris* (colchicine-metaphase). × 2850.

Fig. 3.—Autoradiograph of anaphase in *Crepis* root cell after 6 hours growth in thymidine-H^3. × 2150.

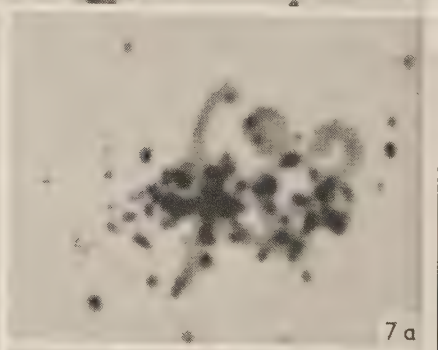

1a
1b
II
I
III
I
2
3

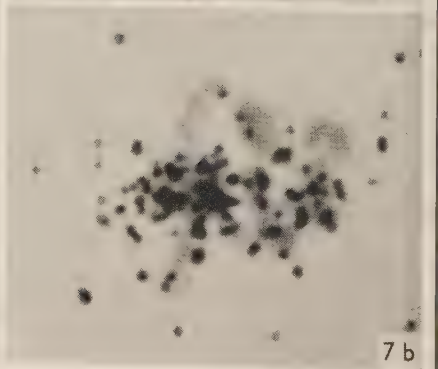
4a

4b


III
I
II
III
III
II
I
5

6a


7a


7b

DISCUSSION

All of the daughter chromosomes are regularly labeled at the first division after incorporation of the thymidine-H^3. After one duplication in the absence of the labeled precursor, all chromosomes yield one labeled and one unlabeled daughter. Therefore, the chromosomes of *Crepis* like those species previously investigated [4, 5] must be two-stranded. The strands separate at duplication and along each is built a new strand which is probably complementary to it. The complementarity of the two strands seems more probable since an analysis of chromatid exchanges in *Bellevalia* [4] showed that the two strands are different in some structural sense that restricts the type of rejoining that can occur among the four strands of two sister chromatids.

The interesting new feature revealed by the chromosomes of *Crepis* is the gradient of labeling that occurs along the chromosome. Since this occurs only in those first chromosomes labeled when the roots are placed in thymidine-H^3, it must be associated with an increase of specific activity of the precursor of the thymine-containing nucleotide of DNA. The gradient can be explained if the specific activity of the precursor is increasing during the time of chromosome duplication. In these circumstances the DNA in the part of the chromosome duplicated first would have the lowest specific activity and the part duplicated last would have the highest specific activity. If duplication of part of the chromosome were complete before the cell came in contact with the thymidine-H^3, the label would be restricted to the last part to be duplicated. On the basis of this reasoning, one may say that the chromosomes of *Crepis* are regularly duplicated from both ends toward the cen-

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Fig. 4 (*a*, *b*). Individual chromosomes from tetraploid c-metaphases (the second division after labeling occurred) which were well flattened against the film. × 2850.

Fig. 5.—Diploid cell at the second division after labeling; exposed to colchicine long enough for the daughter chromosomes to separate completely. Note that only one of each daughter pair is labeled. × 2850.

Fig. 6.—Autoradiograph of prophase chromosomes from a cell grown for 6 hours in thymidine-H^3. Note the gradient of labeling along each of the six chromosomes. (*a*) Photograph of the grains. (*b*) Sketch to show the position of the chromosomes under the grains; approximate position of centromeres marked. × 1600.

Fig. 7.—Metaphase in a cell grown for 6 hours in thymidine-H^3; note concentration of label in the region of the centromeres which are oriented on the metaphase plate. (*a*) Lower focal level; (*b*) upper focal level. × 2850.

Fig. 6 b.

tromere and a long arm requires a longer period for duplication than a short one. Duplication of an arm proceeds to the centromere and stops. This is illustrated by the upper chromosome I in Fig. 6*a*. The two arms in this instance began to duplicate at about the same time, for the end of the long arm is labeled to about the same extent as the short arm. The proximal portion of the long arm was duplicated at a later time when the specific activity of precursors was higher. Duplication of the chromosomes appears to be synchronized to some degree but variations between individual chromosomes occur. The gradient noted cannot be due to a gradient of DNA concentration, because the labeling gradient occurs only in the first chromosomes labeled after placing the roots in thymidine-H^3 solutions.

Three analogies may now be drawn between chromosomes and the Watson-Crick model for DNA, namely: (1) both are two-stranded; (2) the two strands are different in both the chromosome and the DNA model, and (3) the chromosome appears to replicate in a "zipper" fashion as the double helix of DNA would presumably have to do. The most obvious difference between the chromosome and the DNA double helix is size or the number of nucleotides. The chromosome even when uncoiled is at least 0.1–0.2 micron in diameter while the double helix is 25 30 Å. If all the DNA were in one double helix, the length would reach a meter in some of the larger chromosomes [3]. This is one to two hundred thousand times the size of the largest molecules of DNA so far isolated. The chromosome is, therefore, probably an array of DNA double helices held together as a unit. Whether the molecules are attached lengthwise or at right angles to the chromosomal axis is yet to be determined. Taylor [3] has suggested a model in which the DNA double helices are attached along the edges of a double chromosomal axis or core. One chain of each double helix would be attached to one strand of the core and the other chain to the opposite strand. The other end of each double helix would be free to rotate at least during duplication.

The apparent discrepancy between the behavior of tritium-labeled chromosomes and those labeled with thymidine-^{14}C by Plaut and Mazia [1] is not altogether clear. However, the explanation originally suggested [5] appears reasonable on the basis of the length of the cell division cycle disclosed by the observations reported here. Cells, grown for 6 hours in thymidine-H^3, 10 hours in a solution free of thymidine, and 8 hours in colchicine, were usually blocked in the third metaphase after labeling. This means that at 25° two and one-half cell division cycles may occur in less than 24 hours. Plaut and Mazia after growing roots 12 hours in thymidine-^{14}C may have seen in some cells the result of two replications in labeled thymidine. The random

distribution of 6 chromosomes in these cells would yield about 1 in 32 pairs of telophase nuclei in which one would have two times as much label as its sister nucleus.

SUMMARY

By the use of thymidine-H^3, DNA of chromosomes in root cells of *Crepis capillaris* was labeled. At the first division following the incorporation of label all chromosomes were labeled. After one duplication in the absence of label these chromosomes produce one labeled daughter and one free of label. This behavior is like that previously described for *Vicia* and *Bellevalia*, and can only be accounted for if the chromosome is two-stranded before duplication. In addition some of the chromosomes of *Crepis* fixed 5–7 hours after the roots began growing in thymidine-H^3 show a gradient in concentration of tritium with the least amount of the isotope at the ends and an increasing concentration toward the centromere. The interpretation is that the chromosomes begin their duplication at the ends with a regular progression toward the centromere.

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SURFACE ANTIGEN DEFECTS CONTRIBUTING TO DEVELOPMENTAL FAILURE IN AGGREGATELESS VARIANTS OF THE SLIME MOLD, *DICTYOSTELIUM DISCOIDEUM*[1]

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Received December 24, 1957

During the growth phase of the life cycle of amoeboid slime molds large numbers of independent-living vegetative amoebae are provided by binary fission. Such amoebae have the ability to respond to a chemotactic substance called acrasin which results in their aggregating to form colonies or pseudoplasmodia. By subsequent morphogenetic processes a mature sorocarp composed of stalk cells and spore cells is formed [1, 2]. Thus cells which had existed independently eventually compose a multicellular organism. Under these circumstances a mechanism may exist which enables the cells to adhere to each other and which allows the freedom necessary to accomplish morphogenetic movements.

Tyler [10] visualized that the adherence of cells of multicellular organisms is effected by forces similar to those involved in an antigen-antibody reaction. The Weiss [12] hypothesis suggests that complementary proteins on adjacent cell surfaces act as units which link the cells together. The surfaces of *Dictyostelium discoideum* wild type amoebae[3] have been shown to undergo a serologically detectable change which occurs as the amoebae approach the aggregation stage. This change has been interpreted as due to the appearance of new antigens which, though not necessarily, have a role in cell adhesion and the coordinated movements of amoebae [4, 11].

Sussman and Sussman [9] by the use of ultra-violet irradiation were able

[1] This investigation was supported in part by a research grant E-1452 from the National Institutes of Health, Public Health Service.

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[3] *Dictyostelium discoideum* wild type amoebae will be referred to hereafter in this paper as *D. discoideum* w.t. for the sake of brevity.

to produce variants of *D. discoideum* which have lost the ability to aggregate. It was of interest, therefore, to investigate such variants serologically in the hope of elucidating further the role of the cell surface in morphogenetic movements and cell adhesion. It is conceivable that clues to the mechanism of normal development may be found by investigations of the arrested amoebae.

METHODS AND MATERIALS

Preparation of injection antigens.—The slime molds, *D. discoideum* w.t. and the *D. discoideum* variants,[1] aggregateless 91, 89, and 73 were cultured according to Bonner [2]. Either *Escherichia coli* or *Aerobacter aerogenes* was used as the bacterial associate. The cultures were grown for approximately 48 hours at 22°C. The concentration of amoebae was adjusted such that 1.0 ml of suspension contained approximately 0.21 mg total N.

Preparation of antisera.—Preinjection (control) sera were taken from the rabbits by heart puncture before the immunizing antigen injections were begun. The blood was allowed to stand at room temperature for about an hour before being placed in a refrigerator overnight. The sera were obtained by centrifugation and stored in serum bottles in a deep freeze. The sera were heated before use to 56°C for 30 minutes in order to destroy complement.

The procedure followed in preparing antisera involved injecting three rabbits each with 0.5–1.0 ml of a particular antigen. Intravenous and subcutaneous injections were alternated until a total of nine injections had been given over a period of 21 days. The rabbits were bled 4 days following the last injection. A variation of the injection procedure involved alternating intravenous, intraperitoneal and subcutaneous injections until a total of nine injections were given over a period of 17 days. The rabbits in this instance were bled 5 or 6 days following the last injection. The antisera were prepared in the same manner as described previously for preinjection sera.

The antibody assay procedure.—The test antigens were prepared by washing (in 0.5 per cent NaCl) approximately 48 hour amoebae of *D. discoideum* w.t., Agg 91, Agg 89, Agg 73, Agg 70 and Agg 53 relatively free of bacteria in the same manner in which the immunizing antigens were prepared. In order to detect the presence or absence of antibodies 10.0 μl of a suspension of amoebae (approximately 1.5×10^8 cells/ml) were placed on a microscope slide. A 10.0 μl sample of antiserum was mixed with the amoebae by means of a small glass rod. A coverslip (No. 1) was placed over the mixture but was supported by two other coverslips which had been paraffined to the microscope slide. The amoebae, therefore, were arranged in a thin film and not inhibited from agglutinating by the pressure of a coverslip upon them (Fig. 1). Agglutination, when observed, resulted in various degrees of clumping of the amoebae. The maximum degree of clumping resulted in large masses or clumps of amoebae

[1] The variants of *D. discoideum* were kindly provided by Dr. M. Sussman, Department of Biological Sciences, Northwestern University, Evanston, Illinois.

which were readily visible macroscopically (Fig. 2*a*). The preinjection serum (control) and antiserum produced in each rabbit were tested separately with respect to their agglutinating capacity on homologous and heterologous amoebae. The results from all sera, however, were compiled in Table II.

It will be stated subsequently in this paper that certain of the aggregateless variants could only be slightly agglutinated in homologous antisera. Such antisera furthermore could not agglutinate *D. discoideum* w.t. amoebae. In order to demonstrate the presence of antibodies in sera which were prepared from these aggregateless variants it was necessary to perform precipitation tests. Such precipitation tests also served as a means of titering all antisera.

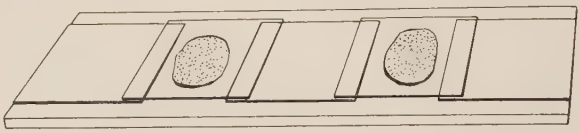

Fig. 1.—Illustrates the type of chamber utilized for agglutination tests of slime mold amoebae.

The test antigens for the precipitation tests were prepared by freezing and thawing one volume of packed cells obtained by centrifuging relatively bacteria-free amoebae for ten minutes at 125 × gravity. Such amoebae were harvested from approximately 48 hour culture plates. The homogenate then was diluted with nine volumes of phosphate buffer (0.01 *M* buffer in 1.0 per cent NaCl at pH 7.4). This preparation was centrifuged for 30 minutes at 29,000 × gravity. The resulting clear supernatant was defined as a 1:10 dilution of antigen from which further serial dilutions of 1:100, 1:1000, 1:2000, and 1:4000 were made.

Precipitation tests were performed by placing approximately 15.0 μl of undiluted antisera or preinjection sera into 3.0 mm I.D. glass capillaries. Approximately 30.0 μl of the serial dilutions of the test antigens were layered over the antisera. The tests were allowed to stand for two hours at room temperature before being examined for the presence of a ring of precipitate. The titers of the various sera are shown in Table I.

TABLE I. *Titers of various undiluted antisera with homologous antigens. Controls of preinjection sera were negative.*

Antisera (undiluted)	Highest dilution of antigen showing precipitation
D. Discoideum w.t.	1:2000
Agg 91	1:1000
Agg 89	1:2000
Agg 73	1:2000

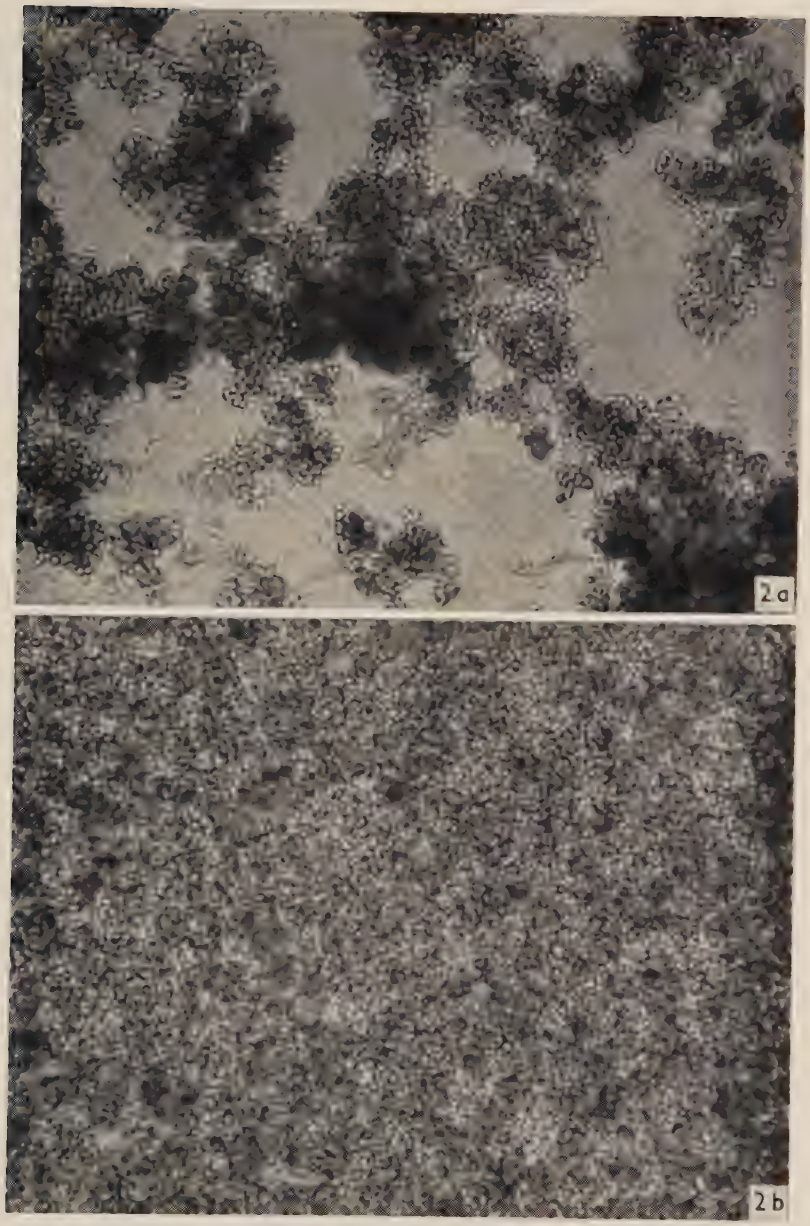

Fig. 2 *a*.—*D. discoideum* w.t. amoebae × anti-*D. discoideum* w.t. serum. (Magnification × 112.)
Fig. 2 *b*.—*D. discoideum* w.t. amoebae × control serum. (Magnification × 112.)

TABLE II. *The degree of agglutination effected by various antisera and preinjection (control) sera on homologous and heterologous types of amoebae.*

+ = maximum agglutination; ± = slight agglutination; and − = no agglutionation. All sera were diluted to one-half of full strength as a result of the addition of amoebae. The numbers opposite the degree of agglutination values refer to the number of times a particular pattern was observed.

Vegetative amoebae	Degree of agglutination	Antisera and preinjection (control) sera (A = Antiserum; C = Control)							
		D. discoideum w.t.		Agg 91		Agg 89		Agg 73	
		A	C	A	C	A	C	A	C
D. discoideum w.t.	+	27						15	
	±								
	−		25	17	17	25	20		15
Agg 91	+								
	±	1	1	15		18			
	−	18	17	7	22	2	15	15	15
Agg 89	+								
	±	2	1	6		20			
	−	30	31	19	25	7	21	17	17
Agg 73	+	17						16	
	±					1	1		
	−		17	15	15	19	14		16
Agg 70	+	7							
	±								
	−		7						
Agg 53	+	8							
	±								
	−		8						

RESULTS

Anti-D. discoideum w.t. serum.—An examination of Table II will show that anti-*D. discoideum* w.t. serum is capable of agglutinating *D. discoideum* w.t., Agg 73, Agg 70, and Agg 53 amoebae to the maximum degree (+). Fig. 2*a* illustrates this particular degree of agglutination.

The *D. discoideum* w.t. control (preinjection serum) affected the wild type Agg 73, Agg 70, and Agg 53 amoebae to a negligible degree (−) (Fig 2*b*).

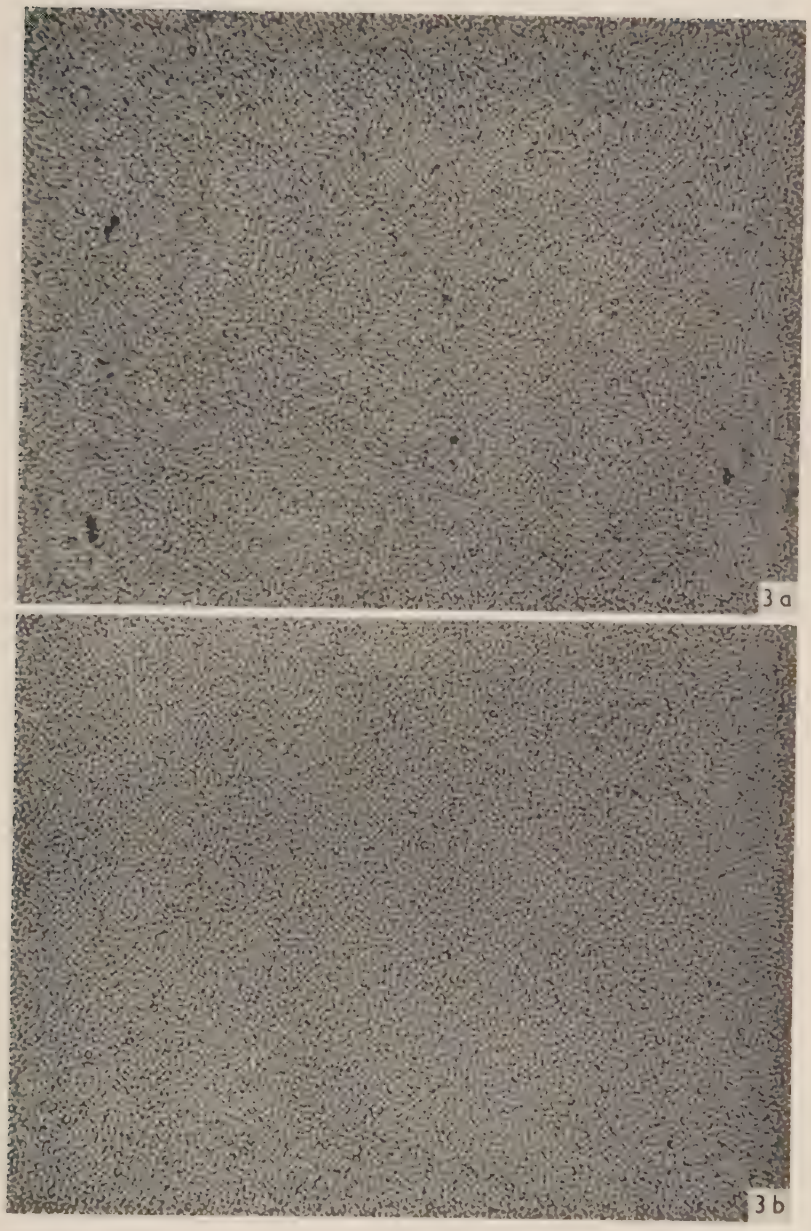

Fig. 3*a*. Agg 89 amoebae + anti-*D. discoideum* w.t. serum. (Magnification × 112.)
Fig. 3*b*. Agg 89 amoebae + control serum. (Magnification × 112.)

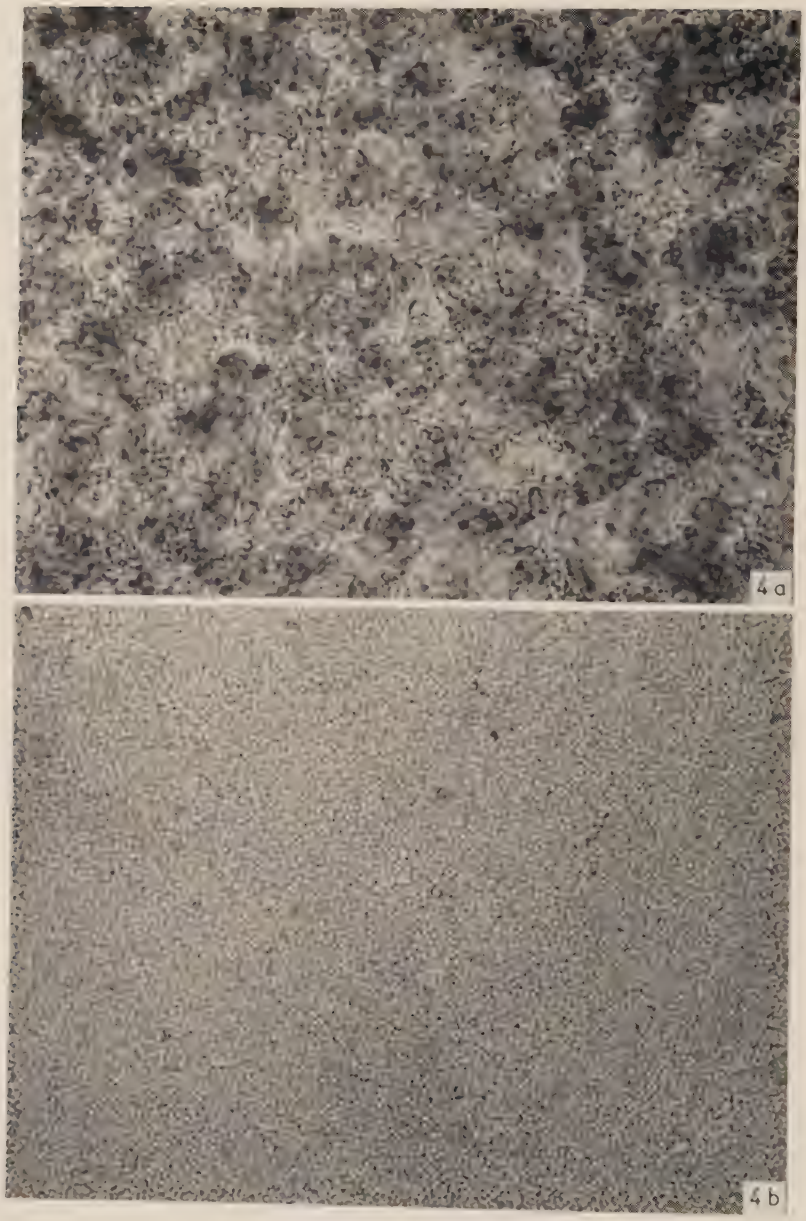

Fig. 4*a*.—Agg 89 amoebae × anti-Agg 89 serum. (Magnification × 112.)
Fig. 4*b*.—Agg 89 amoebae × control serum. (Magnification × 112.)

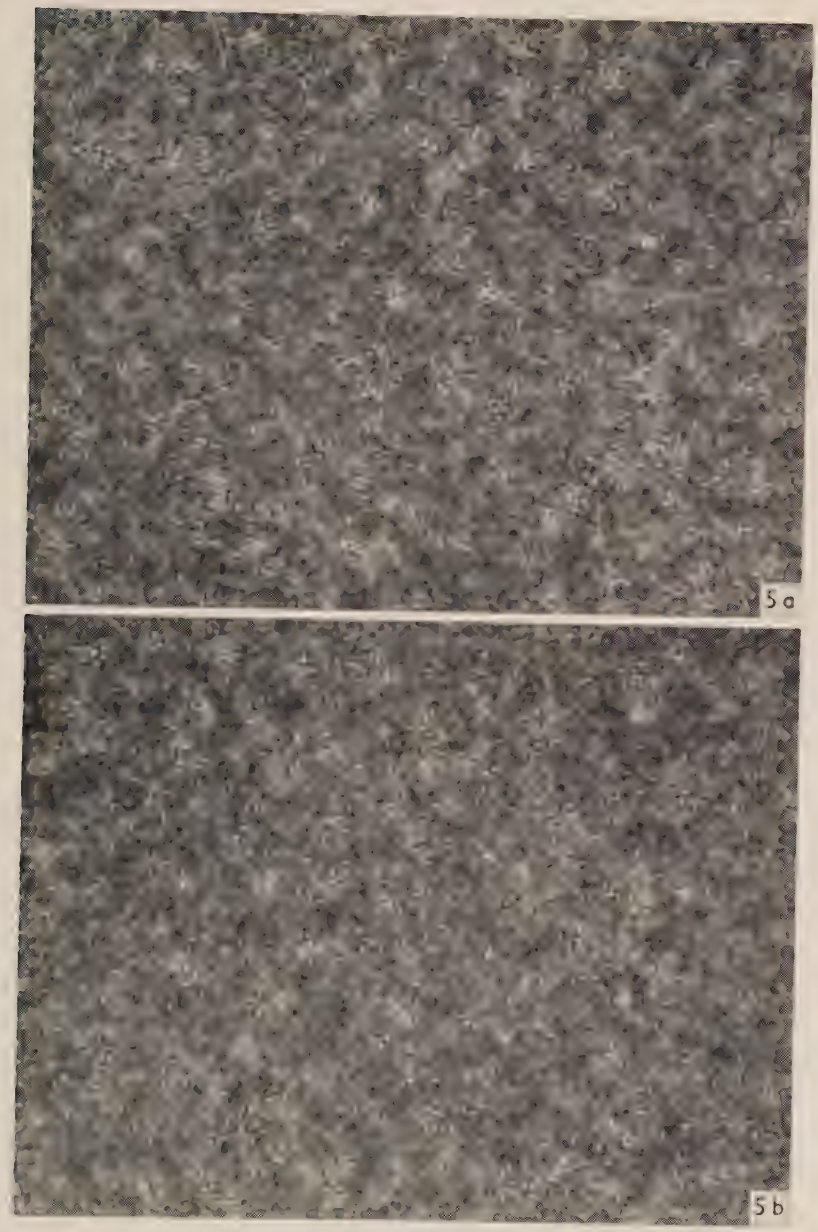

Fig. 5*a*.—*D. discoideum* w.t. amoebae × anti-Agg 89 serum. (Magnification × 112.)
Fig. 5*b*.—*D. discoideum* w.t. amoebae × control serum. (Magnification × 112.)

It was of interest to determine, however, that anti-*D. discoideum* w.t. serum cannot agglutinate Agg 91 or Agg 89 to a degree greater than that effected by the control (preinjection serum) (Table II). The greatest effect observed was a slight agglutination (+). The typical patterns observed, however, are illustrated in Fig. 3*a* and 3*b*. Thus the conclusion is reached that aggregateless variants Agg 91 and Agg 89 could not be agglutinated by anti-*D. discoideum* w.t. serum.

Anti-Agg 91 serum.—Table II discloses that anti-Agg 91 serum is capable of agglutinating Agg 91 and Agg 89 amoebae to a slight degree. Neither *D. discoideum* w.t. nor Agg 73 amoebae was agglutinated by anti-Agg 91 serum.[1]

The Agg 91 control (preinjection serum) did not agglutinate *D. discoideum* w.t., Agg 91, Agg 89, or Agg 73 amoebae.

Anti-Agg 89 serum.—This antiserum acts in a manner identical to that of anti-Agg 91 serum in that Agg 89 and Agg 91 amoebae were slightly agglutinated while *D. discoideum* w.t. and Agg 73 amoebae could not be agglutinated (Table II) (Figs. 4*a* and 5*a*).

Agg 89 control (preinjection serum) did not cause agglutination of *D. discoideum* w.t., Agg 89, Agg 91 and Agg 73 amoebae with the exception of one test involving Agg 73 amoebae in which a slight agglutination occurred (Figs. 4*b* and 5*b*).

Anti-Agg 73 serum.—It may be seen in Table II that anti-Agg 73 serum agglutinates to the maximum degree *D. discoideum* w.t., and Agg 73 amoebae but fails to agglutinate Agg 91 or Agg 89 amoebae.

Agg 73 controls (preinjection serum) did not agglutinate *D. discoideum* w.t., Agg 91, Agg 89 or Agg 73 amoebae.

DISCUSSION

In an attempt to determine the role of the surface antigens in cell adhesion and in effecting morphogenetic processes several ultra-violet induced variants of *D. discoideum* w.t. were employed in this investigation [9]. Certain of these variants lack the ability to aggregate or to produce the stimulus which initiates aggregation [5]. Sussman and Lee [8] have shown that certain aggregateless variants if separated from aggregating *D. discoideum* w.t. amoebae by a thin agar membrane would tend to congregate in small numbers opposite the most active acrasin producing regions. Ennis [3] utilizing the same technique has shown that the degree of response to *D. discoideum* w.t. across the membrane

[1] Unfortunately Agg 70 and Agg 53 cultures were lost before agglutination tests were completed.

ranged from a "strong attraction" Agg 70) to "no sensitivity to wild type" (Agg 91 and 89). However, under these conditions even the sensitive aggregateless could not realize further development. Ennis [3] has shown that acrasin similar to wild type acrasin was present in Agg 91 and Agg 70 variant amoebae. It would appear that failure of the various aggregateless variants to complete development cannot be due to the lack of acrasin. Agg 91, Agg 89, Agg 73, Agg 70, and Agg 53 have the ability to enter into the aggregate and to complete the normal fruiting process when mixed with *D. discoideum* w.t. or certain fruitless variant amoebae [3, 5, 6]. Although relatively small numbers of *D. discoideum* w.t. amoebae initiated aggregation in the variants the ultimate fruiting bodies were composed of more or less equal numbers of *D. discoideum* w.t. and aggregateless variant amoebae [7]. Therefore, the variant amoebae were ensured of surface contacts with *D. discoideum* w.t. amoebae during morphogenetic movements. It appears that surface contacts between *D. discoideum* w.t. or certain fruitless variants and aggregateless variants are essential for the variants to regain their normal morphogenetic capacity. It is possible that in some manner the wild-type reconstitutes the cell surfaces of the variants with components necessary to promote normal development. It was of interest, therefore, to determine whether the physiological defects observed in the aggregateless variants could be attributed to surface antigen aberrances detectable by immunological methods.

It is obvious from an examination of Table II that the surface antigens of Agg 73, Agg 70 and Agg 53 amoebae appear to be similar to those of the *D. discoideum* w.t. amoebae with respect to their agglutination patterns induced by anti-*D. discoideum* w.t. serum. It is also apparent that Agg 73 amoebae, which may be agglutinated by anti-*D. discoideum* w.t. serum, produce an antiserum which is capable of agglutinating homologous and *D. discoideum* w.t. amoebae to a maximum degree. However, anti-*D. discoideum* w.t. or anti-Agg 73 sera failed to agglutinate Agg 91 and Agg 89 amoebae indicating that the surface antigens of the latter two variants have been altered or eliminated such that agglutination could not be effected.

Further evidence that the surface antigens of Agg 91 and Agg 89 amoebae normally involved in the agglutination phenomenon were altered or eliminated was revealed by the use of anti-Agg 91 and anti-Agg 89 sera. These sera act identically in that they are capable of effecting slight reciprocal agglutionations of Agg 91 and Agg 89 amoebae but fail to agglutinate *D. discoideum* w.t. or Agg 73 amoebae. Therefore, these amoebae, incapable of being agglutinated by anti-*D. discoideum* w.t. or anti-Agg 73 sera, produce antisera which lack the antibodies necessary to agglutinate *D. discoideum* w.t. or Agg 73

amoebae. From an examination of Table I it is obvious that the amoebae of these types (*D. discoideum* w.t., Agg 91, Agg 89, and Agg 73) produce antisera of similar and reasonably high titer with homologous antigen. Therefore, the aggregateless forms Agg 91 and Agg 89 are antigenic when injected into rabbits. However, it appears that the particular antigens necessary to effect maximal agglutination are missing from their surfaces. In this event neither Agg 91 nor Agg 89 could produce antiserum capable of maximally agglutinating homologous or heterologous amoebae. The agglutination effected by anti-*D. discoideum* w.t. serum on *D. discoideum* w.t., Agg 73, Agg 70, and Agg 53 amoebae may be attributed to the presence of antigens responsible for such agglutinations. Although agglutination may be effected in these particular aggregateless variants it does not necessarily signify that the normal complement of surface antigens is present. Whether the agglutinating antisera have been produced in response to those particular antigens involved in cell adhesion and in effecting morphogenetic movements remains problematical.

It is suggested, however, that the failure of aggregateless variants to undergo normal development is associated with the alteration or elimination of certain surface antigens in the amoebae. Such aberrations could conceivably result in misdirected or uncoordinated morphogenetic movements of the amoebae. Under such conditions fruiting body formation could hardly be realized. However, such an interpretation does not preclude the existence of metabolic or other abnormalities which could also contribute to developmental failure.

SUMMARY

1. The procedure for obtaining antisera to wild type and variant strains of the slime mold *Dictyostelium discoideum* was described.

2. Anti-*D. discoideum* w.t. serum was found to maximally agglutinate *D. discoideum* w.t., Agg 73, Agg 70, and Agg 53 amoebae but failed to agglutinate Agg 91 and Agg 89 amoebae.

3. Anti-Agg 73 serum effected maximal agglutination of Agg 73 and *D. discoideum* w.t. amoebae but failed to effect agglutination of Agg 91 or Agg 89 amoebae.

4. Anti-Agg 91 serum was found to slightly agglutinate Agg 91 and Agg 89 amoebae. However, such serum failed to agglutinate *D. discoideum* w.t. and Agg 73 amoebae.

5. Anti-Agg 89 serum proved to be similar in activity to Agg 91 serum.

6. It was suggested that the failure of the aggregateless variants to complete development is associated with aberrant surface changes.

The aid and advice received from Dr. John Brookbank during the course of this research is gratefully acknowledged. Furthermore, the research assistance provided by Mr. Karl Barden contributed immeasurably to this investigation. The authors also wish to acknowledge Dr. Ruth S. Cooper for her critical review of this manuscript.

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Experimental Cell Research **15**, *370–383 (1958)*

CINÉ-MICROGRAPHIC STUDIES ON MITOSIS IN ENDOSPERM

V. FORMATION OF THE METAPHASE PLATE[1]

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Received December 24, 1958

In the previous papers of this series [1, 4] the course of mitosis was analysed by means of ciné-micrographic methods, with special emphasis on the chromosome movements. However, numerous questions, especially those connected with chromosome movements before the beginning of anaphase, were either not studied at all or were only examined from certain aspects.

In the attempt to penetrate some of these problems a detailed analysis was made of several cells recorded on the film. The materials and the methods were the same as those previously described [2].

OBSERVATIONS

The present observations show that, in general, three phases may be distinguished between the end of the contraction stage [3] and the start of anaphase (Fig. 1). These phases are not sharply defined and gradually merge one into another. They vary from cell to cell, both with regard to the duration and to the ease with which they can be distinguished, but they were observed in all species. The phases are distinguished on the basis of the chromosome activity inside the spindle and by the fact that different types of movements show their maximum activity in different phases.

Phase I—early prometaphase follows the contraction stage and does not usually last long, less than 1 hour in *Haemanthus* (Fig. 1, approximately

[1] This paper, as well as Nos. III–IV of the series, is concerned mainly with the analysis of the film Mitosis in Endosperm, made by A. Bajer and J. Molè-Bajer (1955). Sequences from this film are here referred to by the numbers printed in the film itself. The author is especially grateful to Dr. J. Boss and Dr. H. B. Fell, of the Strangeways Research Laboratory, Cambridge, and to Dr. G. Östergren, of the University of Lund, for most valuable suggestions and much help during the preparation of manuscripts of this series (III–V). The author would like also to express his gratitude to Prof. H. Teleżyński of Warsaw University, for valuable criticism and discussion of several problems.

30 mins). This stage may be so brief as to be almost non-existent in cells with vigorous prometaphase movements, so that the contraction stage seems to be followed by phase II. During phase I the kinetochores are motionless or move slowly towards the equator of the spindle, but not towards the spindle pole; the chromosome arms straighten towards the pole without making the abrupt movements seen in later stages, and the kinetochores stretch [3] during this stage. In time all these types of movement become more vigorous and phase II gradually begins.

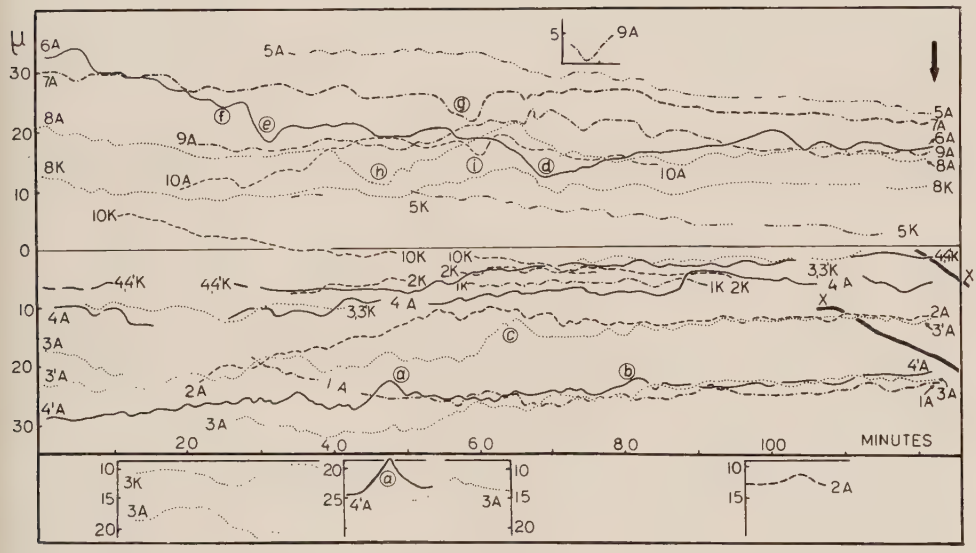


Fig. 1.—*Movements of kinetochore and chromosome ends in prometaphase and metaphase.* The distance of kinetochores (*K*) and ends (*A*) from the approximately estimated equatorial plane (*O*, cf. Fig. 4*e*) plotted against time. The paths of the kinetochores and the ends are drawn in Fig. 2. The contraction stage began about 10 mins before 0 time. Between the contraction-stage anaphase three phases in the movement are discernible. Phase *I*, early prometaphase; chromosome movements begin, the limbs and the kinetochores moving slowly; approx. 30 mins on graph. *II*, mid-prometaphase; vigorous kinetochore and arm movements which cause the arrangement of the chromosomes during the change from prophase to metaphase; approx. from 30 to 90 mins on graph. *III*, metaphase, only slight movement of chromosomes in the spindle; from approx. 90 mins on graph till beginning of anaphase. Note that the chromosomes at the metaphase plate do not stop their vigorous movements simultaneously. Some abrupt bending movements of the ends are marked by letters in circles (half-circles in Fig. 2); kinetochores and the arms of the same chromosome are marked by the same number. If the movements of the two ends of the same chromosome are measured they are distinguished by a superscript. Arrow—beginning of anaphase. Small graphs above and below: movements of the points on the arm between the tip and the kinetochore. Above, 9*A*, about 10 μ from the end; below, 3*A*, about 12 μ from the kinetochore (the distance from the arm end was difficult to measure exactly, but it was approx. 13 μ); 4′*A*, about 10 μ from the end; and 2*A*, about 10 μ from the end. Notice the path of the abrupt bending movements, compare with Fig. 2. *Haemanthus* cell 329, film cell No. 37. ×, ×—directed movement towards the pole in anaphase of the small particles previously in Brownian motion.

Phase II: mid-prometaphase.—This is the period of vigorous kinetochore and arm movements which leads to the formation of the metaphase plate (Fig. 1, approx. from 30 to 90 mins). Unlike the first and the third, this phase is present in all cells and species, though the movements differ in intensity. The kinetochores move to the equator of the spindle or they may also move towards the pole and then return to the plate. The limbs continue to straighten and show a strong tendency to arrange themselves parallel to the long spindle axis. Peculiar arm movements ("abrupt bending movements", cf. p. 376) are most common at this stage. If persistent nucleoli are present, they pass towards the pole and their elimination is continued in phase III, whereas they may in phase I also be eliminated across the spindle. Gradually all types of movement decrease and phase III begins.

Phase III: metaphase.—The chromosomes are arranged in a more or less regular metaphase plate with most of the arms placed parallel to the long axis of the spindle (Fig. 1, appr. 90 mins starting time). The changes in the chromosome arrangement during this stage may be so slight that they can usually be seen only upon film projection, in contrast to the two previous phases which are detected without difficulty by direct observation. The kinetochores and the arms may oscillate, the former occasionally moving to the pole and returning to the plate. "Abrupt bending movements" of the arm-ends are absent or very rare, but often apppear again before the start of anaphase. The duration of this phase is very variable. In all species both very brief metaphases (in *Haemanthus* a few minutes) and very long ones (in one cell of *Haemanthus* over 3 hours) were found, but in *Haemanthus* metaphase usually lasts 20 to 40 mins. The analysis of the conditions that bring about anaphase is difficult and the available data permit no conclusions. Some general suggestions concerning the physiology of metaphase have, however, been made [4].

Kinetochore Movements

During prometaphase, the kinetochores and the arms execute very complicated movements which may be divided into some extreme types on the basis of their course. Between some, but not all, of them there seems to be a continuous transition (cf. p. 373). Four extreme types of movement may be distinguished.

Movements parallel to the spindle or obliquely across it towards the equatorial plate.—These movements are most common in phase II when their velocity is of the order 1–2 μ/min in *Haemanthus* and *Clivia*, but in phase III they are considerably slower, and have sometimes completely ceased. In some

cells the movements during stage II may be extraordinarily rapid, exceeding 3 μ/min in *Clivia*. The kinetochores may move quickly in this way towards the plate provided their paths miss other chromosomes.

Oscillatory movements.—Very slight and usually parallel to and seldom across the long axis of the spindle. These are most common in metaphase (phase III) though occasionally seen in phase I. Between these movements and those described above and below, a continuous transition seems to exist.

Movements towards the pole during prometaphase and metaphase were observed in species with chromosomes of different sizes. During the movements, the guiding role of the kinetochore is evident. Usually the chromosome moves towards the pole, executes some oscillations and then turns. The turn is very often through 180°, i.e. the same part of the kinetochore that was leading the chromosome to the pole leads it back to the plate; this is indicated by the arrangement of the chromosome limbs which are usually stretched towards the plate during the movement towards the pole, but towards the pole during the movement to the plate. This phenomenon, however, is only observed in those cells in which there is enough space to execute such a turn. The paths of the kinetochore movements to the pole are usually parallel to the long axis of the spindle, and rarely across it. In most cells the paths are different towards the pole and towards the plate, but in some cells, in which a kinetochore executes repeated small movements, the paths are exactly the same. A kinetochore may move a short distance towards the pole, or almost the whole way. Only a small percentage of chromosomes move over long distances, and they are not found in all cells. More than 3 chromosomes were never seen moving to the pole in the same cell and usually there was only one. A continuous transition exists between these movements and those described above and it seems probable that their mechanism is the same.

Revolving movements.—Revolution of the kinetochore is very rare, and two kinds of turn were observed.

(1) The kinetochore turns through 180°, the direction perpendicular to the long axis of the spindle being the axis of revolution. As a result of this the orientation of a kinetochore directed towards the pole is changed towards the plate. Some of the chromosomes moving towards the pole, as previously described, execute such movements.

(2) The kinetochore turns through various angles, the long axis of the spindle being the axis of revolution. This type of movement is described below (p. 374).

Rotation of kinetochores, especially round the long axis of the spindle, seems to have a quite different mechanism from that of the first three types described above, which may be due to traction.

The problem arises whether only a few kinetochores turn around and move towards the pole or whether many do so. In animal and plant material all chromosomes are not arranged in one plane and are also not visible during observation and film projection; in endosperm, however, this limitation applies to certain cells, and in most flattened and half-flattened cells, all the chromosomes are seen wholly or partially. Sometimes it is even possible to count the chromosomes, and the analyses of photographic sequences refer almost exclusively to such cells.

The Limb Movements

As with kinetochore movements, various types of arm movement may be distinguished, between some of which there may be a continuous transition; the mechanism of some kinds of movement may be similar to the mechanism of kinetochore movements.

Revolving movements.—In prophase sister chromatids are coiled around each other (relational coils). This is seen especially clearly in the long chromosomes of *Haemanthus* and *Leucojum:* in prophase of *Haemanthus* some of the long arms have up to 4 or 5 coils. In metaphase, however, the chromatids of a few chromosomes are twisted. Usually the chromatids unwind slowly and even at the highest acceleration (1.5 records/min) the process is not noticeable during projection, but in some cells the revolution is clearly visible. In the long arms of *Haemanthus* the speed of the revolution calculated from the reduction of five coils to none is of the order 0.2 revolution/min. In *Clivia* usually several chromosome arms turn around during the contraction stage. Unfortunately, though the revolving is visible, it was impossible to find out whether this is connected with the uncoiling or the coiling of the sister chromatids. The rapid uncoiling visible during projection occurs in different phases. In *Haemanthus* it occurs after the partial arrangement of the chromosomes at the plate and is most common in middle prometaphase or the beginning of metaphase. The kinetochores seldom turn during the uncoiling of the arms, though they may execute other movements; in a few examples, however, a kinetochore was seen to revolve through 180° 270°, the long axis of the spindle being the axis of revolution. This was observed in *Haemanthus*, in which the kinetochores of some large chromosomes gave evidence of activity before rotation, by making active movements towards the pole and the plate.

Oscillatory movements of the arm parallel to the long axis of the spindle are similar in character and duration to the oscillations of the kinetochores. The arms may also oscillate across the spindle; these movements across the spindle are observed most often in phase III, but they are also often executed in anaphase by the arms that are directed towards the spindle equator.

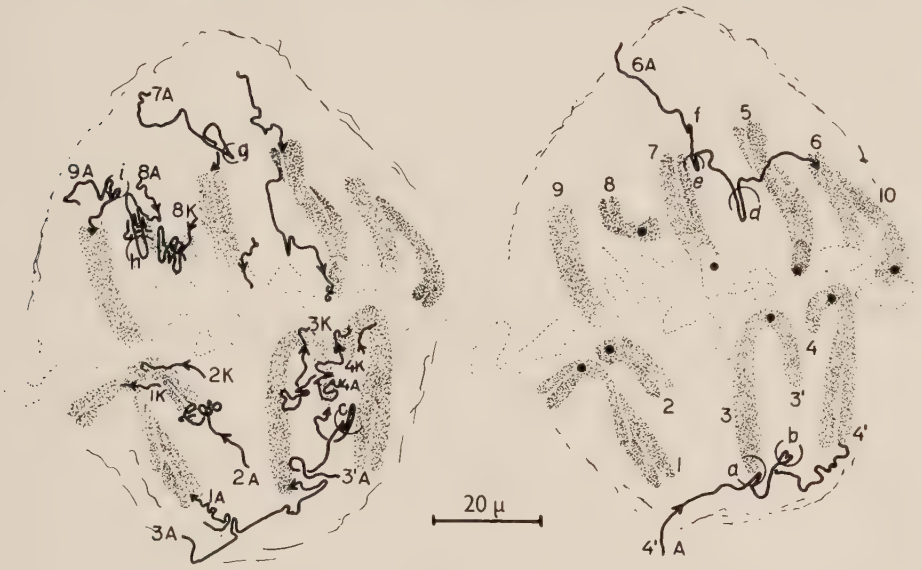


Fig. 2.—*Drawings of the paths of the chromosome ends and of the kinetochores in prometaphase.* Graphs given in Fig. 1.

Straightening of the arms.—This consists of slow movements mostly found in phases I and II and rarely in phase III. As the result of these movements the curved arms (prophase and contraction-stage arrangement) straighten with a very strong tendency to arrange themselves parallel to the long axis of the spindle and probably parallel to its submicroscopic fibrous structure. The tendency of such arrangement to occur was described previously by Östergren [6] as factor δ. In unflattened cells of *Haemanthus* during metaphase, chromosome arms may show slight convergence to the poles, which seems to support this statement. This tendency was noted in plants with medium and large chromosomes, but in those with very small chromosomes it is very difficult to distinguish, owing to the small size of the chromosomes. The direction of straightening is always towards the spindle pole and no movement of this type towards the plate was observed. The process of straightening may be carried out in one or several steps separated by various time intervals.

During the straightening the kinetochores may be motionless or they may move in different directions. Simultaneously with straightening, the chromatids may uncoil and execute revolving movements of the type already described.

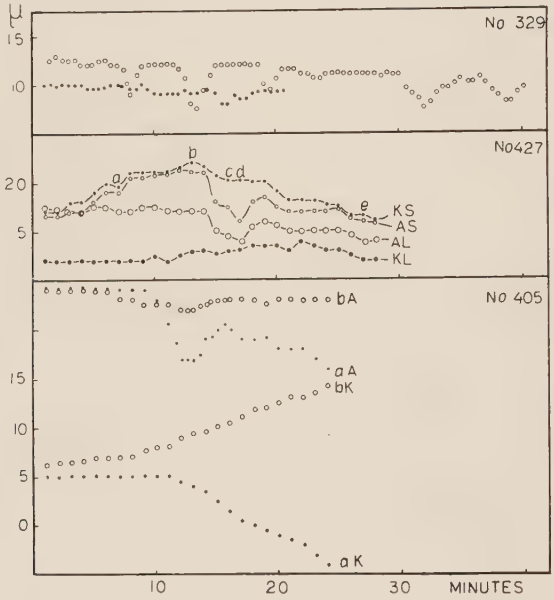


Fig. 3.—*The abrupt bending movements in metaphase of Clivia* (cell No. 329, film cell No. 23) *and in prometaphase* (cell No. 427) *and anaphase of Haemanthus* (cell No. 405, film cell No. 26, cf. Fig. 5). The distance of the chromosome ends and the kinetochores from the approximately estimated equatorial plate, plotted against time. One chromosome arm in *Clivia* (white circles) makes repeated movements, while the second does not.

In prometaphase of *Haemanthus* the movement of chromosome ends (*A*) and kinetochores (*K*) of a large (*L*) and a small (*S*) chromosome is measured. These ends lie with the spindle pole in one straight line; cf. drawings in Fig. 4. In anaphase the movement of two sister chromosome ends (*aA*, *bA*) and that of the kinetochores (*aK*, *bK*) are marked. While one of the ends (*aA*) makes an abrupt bending movement, the second does not, cf. Fig. 5.

The abrupt bending movements. –This type of movement is a puzzling one and deserves special attention. Apart from the slow straightening the arms may bend abruptly towards the plate, this being always followed by the arm straightening (prometaphase); the movement is so sudden, in comparison to other movements, that it gives the impression of a sudden shock applied to the arm (Figs. 1, 2 and 3). The occurrence of such bending was previously reported [4], but no exact analysis was made. These movements are noticed mainly in phase II, after which they stop temporarily to reappear at the end

TABLE I. *Abrupt bending movements (a.b.m.) in* Haemanthus Katharine.

Number of a.b.m. per cell	Stage: number of cells			
	Prometaphase	Metaphase	Anaphase* a ∨	Anaphase* b ∧
0	2	38	40	39
1	3	10	4	3
2	3	1	7	3
3	5	0	3	3
4	5	0	0	2
5	4	0	0	2
6	5	0	0	0
7	2	0	0	0
8	3	0	1	0
9	4	0	0	0
10	0	0	0	0
11	0	0	0	1
12	2	0	0	1
13	0	0	0	0
14	1	0	0	0
15	1	0	0	1
16	0	0	0	0
17	1	0	0	0
18	2	0	0	0
25	0	0	0	1
36	1	0	0	0

* a ∨, Movements towards the equatorial plane executed by arm ends directed to the pole (placed in half-spindle). b ∧, movements towards the spindle pole executed by arm ends directed to the equatorial plane (placed in the interzonal region).

of metaphase; they are rare in anaphase. Their occurrence is illustrated by Fig. 6 and Table I.

These peculiar movements are executed by chromosome arms of large and medium size, being most clearly seen in *Haemanthus*, *Leucojum* and *Clivia*. In all the plants studied, the course of the abrupt bending movements is exactly the same if the movement is not disturbed by mechanical conditions.

In the abrupt bending movements two distinct phases, one always following the other in the same order, may be distinguished: (1) movement from the pole, and after a short interval, (2) movement towards the pole. The same arm may execute two, three or even more abrupt bending movements with

different time intervals (Figs. 1, 3 and 5) and sometimes without any intervals between one movement and the next; in this latter case the arm is bent towards the plate, partly or completely straightened, immediately bends again and then straightens once more. Such a course of movement is seen best

Fig. 4.—Drawings of the position of the chromosome whose graphs are in Fig. 3. Line *e*, approximately estimated equatorial plane. *Haemanthus* cell No. 427, letters *a*, *b*, *c*, and *d* as in Fig. 3.

during observations of the arm ends, though an end may remain motionless while the middle of the arm executes the abrupt bending movements. Efforts were made to find out whether there are any differences between the movement towards the plate and that towards the pole except those of direction, but if the differences exist they cannot be revealed by the present analysis. In many abrupt bending movements the velocity towards the plate is higher than towards the pole, but the contrary was also observed. In *Haemanthus*, the force that bends the arm may act either on the end of the limb, or on another part of the arm between the tip and the kinetochore up to 15 μ from the tip (Fig. 1, small graphs $3A$, $4'/A$, $2A$ and $9A$); the length of the long

arms of large *Haemanthus* chromosomes is about 25 μ. At present it is not known whether the particles in Brownian motion may also move in the same way as the chromosome ends during abrupt bending movements.

In anaphase the abrupt bending movements occur in the half spindles, executed by arms moving in front of the kinetochores, and are similar to those in prometaphase and metaphase described above; the arms directed towards the spindle equator may also perform these movements at this stage (Table I). In the latter case, the arms first execute the movements towards the spindle pole, i.e. in the direction of the moving chromosomes, this being immediately followed by the movement towards the spindle equator. The force responsible for the second part of the movement (that to the equator) may be so strong that the chromosome arms are slightly, but visibly, stretched; they contract in a few minutes to their former length. It is very difficult to analyse the details of some of the abrupt bending movements of the last kind. It seems probable that occasionally the movement towards the equator occurs first, followed by that towards the pole, but this is not certain.

The problem arises as to what kind of movement previously described these bending movements are comparable to. It seems probable that they are most similar to certain oscillations and unlikely that they are closely related to the straightening of the chromosomal limbs.

The abrupt bending movements may be caused by structural changes in the spindle, and if so we must consider which part of the spindle is responsible for exerting the necessary force. During the bending of one arm, the second arm, which may be less than 5 μ, distant (the two arms are parallel to the long spindle axis), may or may not perform similar movements. Where the second arm also executes an abrupt bending movement it is usually much weaker than that of the first, and often there is a delay between the beginning of movement in the two. Where the arms lie at different levels on a line parallel to the long axis of the spindle (Fig. 4), the arm placed nearer to the plate nearly always moves slightly later and usually more weakly. The time of "conduction" of the movement from one arm to the other often corresponds to the time which the moving arm would have taken to cover a similar distance. Very often also the movement of two such arms seems to start simultaneously, or if a difference in time exists it is not measurable. No example was found of an arm placed nearer to the plate beginning the movement earlier, which suggests that the force of the first part of an abrupt bending movement acts from the pole. Comparison of the movements of neighbouring chromosomes indicates that there is a centre where the acting force is strongest and from which it may act in different directions, albeit unequally.

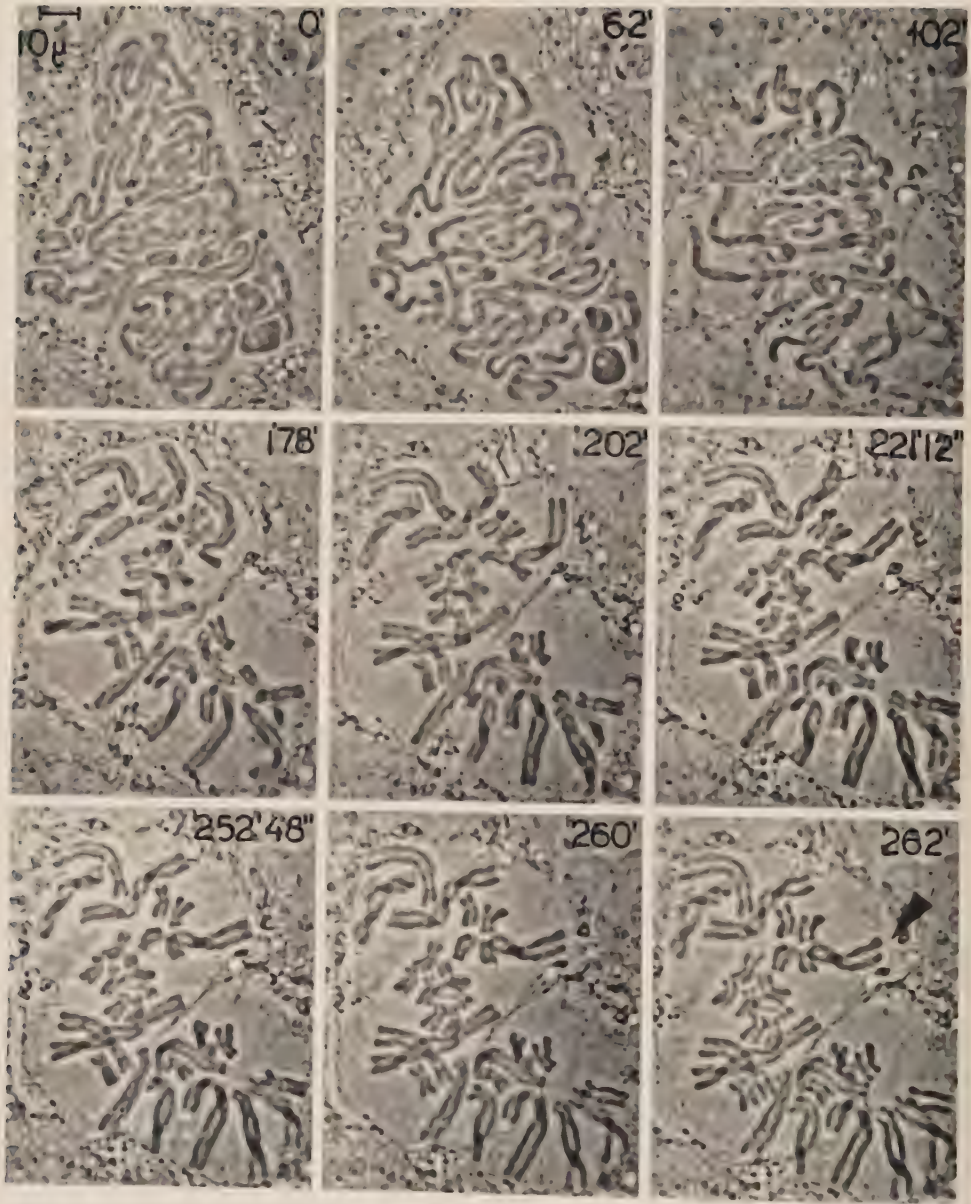
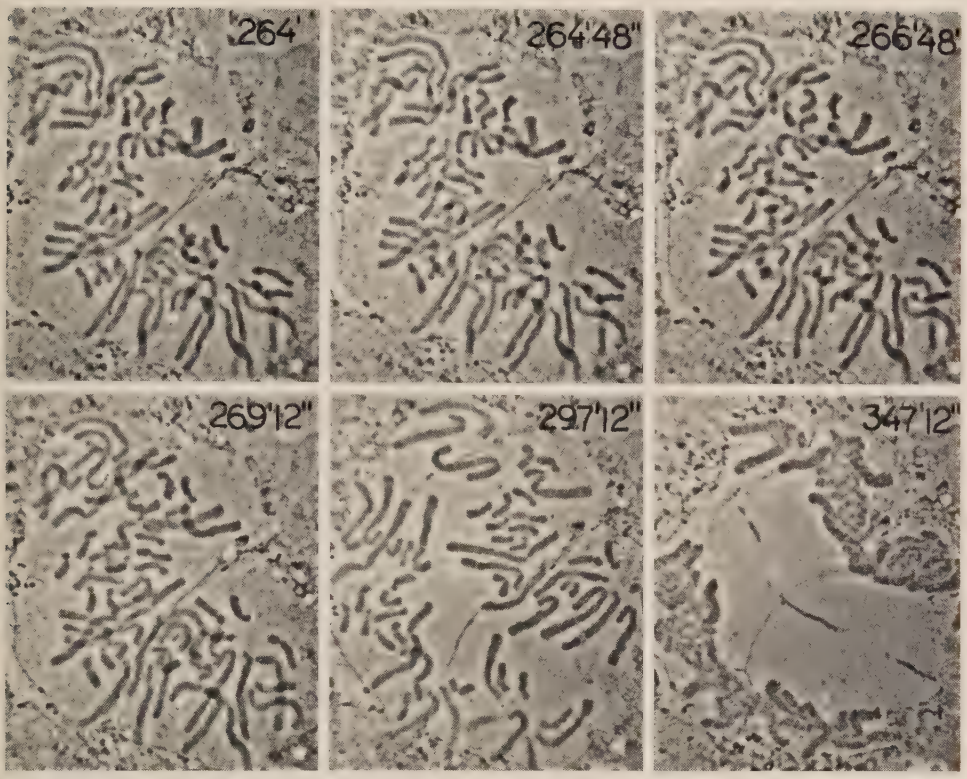


Fig. 5.—Mitosis in an experimentally flattened cell of *Haemanthus*. Spindle formation (0'–62') and abrupt folding movements (202'–260') during anaphase. The graph of this movement is shown in Fig. 3. The time is given in micrographs. Cell No. 405, film cell No. 26.



DISCUSSION

Kinetochore activity inside the spindle is manifested in different kinds of movement and in stretching of the kinetochores. The stretching was observed immediately after the contraction stage [3] and during this process the thickness of the kinetochores increases and then diminishes. From the theoretical point of view two types of movement are important: those more or less across the spindle and revolving movements. Since the hypothesis that chromosome movement is partly controlled by the activity of the spindle is accepted by most authors, special attention should be drawn to the lability of the connection between the kinetochore and the spindle. In the movement of the kinetochore towards the pole the guiding role of the former is evident, though the mechanism by which the kinetochore turns towards the plate in prometaphase is not yet understood.

One of the most characteristic features of prometaphase are the movements

of the chromosome limbs. Between some types of movements there seems to be a continuous transition and their mechanisms are probably identical. The question arises, however, whether all types of movement have a similar mechanism. This question cannot be answered at present, though it seems probable

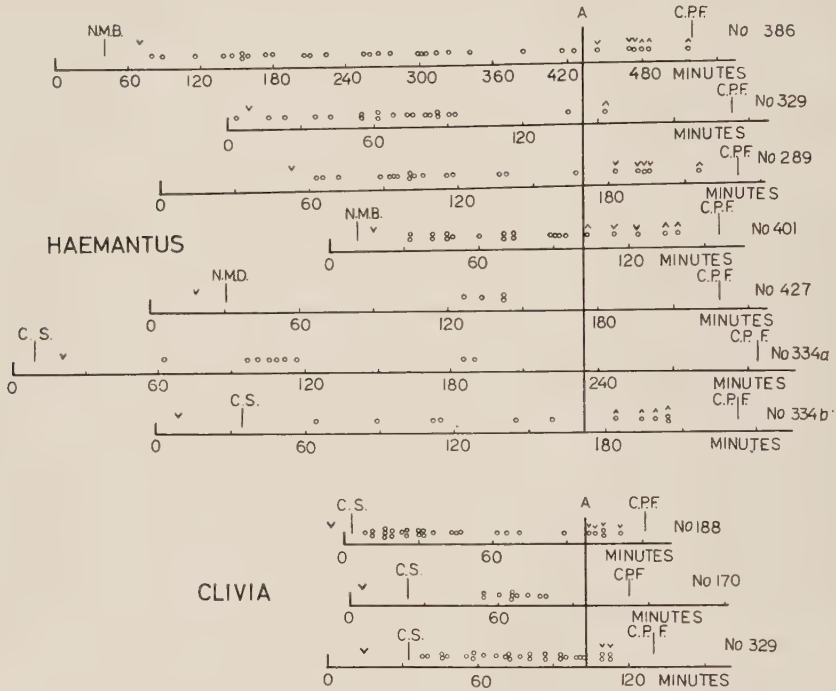


Fig. 6.—*The occurrence of the abrupt bending movements in the mitosis of different cells.* The duration of prometaphase and metaphase varies in different cells; the abrupt bending movements usually appear some time (10–60 mins) after the disintegration of the nuclear membrane and the end of the contraction stage [3]; three periods in their occurrence may be distinguished: *I*, between contraction stage and mid-prometaphase when abrupt bending movements begin; *II*, mid-prometaphase, when they are most frequent; *III*, metaphase in which they are usually much less common. Direction of movement: V, movements towards the plate; ∧, movements towards the pole. Until the beginning of anaphase only the former were observed. *N.M.B.*, nuclear membrane breaking; *C.S.*, beginning of contraction stage; *A*, beginning of anaphase; *C.P.F.*, cell plate formation. The distribution of abrupt bending movements is shown in Table I.

that in the arm movements at least three mechanisms or forces are involved causing (1) kinetochore movements, (2) slow straightening of the arms, and (3) the abrupt bending movements. These last seem to be hardest to explain, but it is also difficult to suggest an explanation of the slow straightening of the arms. The abrupt bending movements, at least the first part, i.e. the movement to the plate, may be due to changes in the spindle and not in the chro-

mosomes. This interpretation is supported by experiments with colchicine [5] under the influence of which there are no abrupt bending movements, although the slow straightening of the arms remains evident. Unfortunately the nature of the changes in the spindle cannot be defined on the basis of the present studies, nor can it be determined whether the abrupt bending movements are the effects of the main mechanism moving the chromosomes or are not strictly connected with it.

SUMMARY

During the formation of the metaphase plate the following types of movement of the chromosome limbs and the kinetochores may be distinguished, but intermediate types may also be found.

1. Kinetochores: (*a*) Movement parallel to or partly across the spindle, (*b*) oscillatory movements, (*c*) movements towards the pole during the prometaphase, (*d*) revolving movements perpendicular or parallel to the long axis of the spindle. It is suggested that the mechanism of (*a*), (*b*) and (*c*) is similar (traction forces) but no explanation for (*d*) is proposed.

2. Arms: (*a*) Revolving movements—uncoiling of chromatids caused by the internal changes in the chromosomes, (*b*) oscillations caused by the kinetochore movements, (*c*) straightening of the arms—no suggestion concerning the mechanism is offered, (*d*) abrupt bending movements—probably passive movements caused by changes of the internal structure of the spindle.

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Experimental Cell Research **15**, *384–397 (1958)*

INDUCTIVE INTERACTION OF EMBRYONIC TISSUES AFTER DISSOCIATION AND REAGGREGATION

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Received December 26, 1957

Metanephrogenic mesenchyme from 11-day mouse embryos has been shown to require an inductive stimulus to differentiate into epithelial tubules *in vitro* [7]. Embryonic dorsal spinal cord is a potent tubule-inductor, and has been used in a series of studies designed to characterize the properties of this interactive system [8–10]. In connection with these studies, questions arose about the importance of tissue integrity in the inductive process. Information was sought through procedures [14] for tissue dissociation and reassociation which have been increasingly widely used in developmental studies in recent years [3, 6, 12, 15, 17, 20, 22, 24].

These procedures have involved various combinations of chemical and mechanical means. For large masses of adult tissues, blenders have been employed in conjunction with chemical treatment, the cell suspension being separated from residual tissue by decanting and screening [19, 25]; for small tissue masses such as are commonly dealt with in studies of vertebrate morphogenesis, dissociation has been accomplished by dispersing through small-bore pipettes tissue treated with trypsin, versene or alkali [12, 13, 26]. These methods are satisfactory for many purposes but they proved neither suitable nor convenient for batches of many extremely small rudiments which are to be dispersed under uniform conditions. The pipette method is laborious and difficult to quantitate, while mechanical mass methods developed for adult tissues require large amounts of tissue and do not yield full disaggregation.

In the present report, a standardizable and potentially quantifiable procedure is described by which pooled embryonic rudiments of small size in fairly large number can be reliably dissociated to varying degrees, including

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reduction to single-cell suspensions. The procedure is also useful for separation of components of embryos and their rudiments, and for prolonged exposure of small tissue masses to dissociative forces of controlled intensity. Using this procedure, it is shown that in the dorsal spinal cord–metanephrogenic mesenchyme system: (1) dissociation of either interactant does not destroy its ability to participate in induction; (2) mixed suspensions of the two tissues produce mixed reaggregates which segregate into type-specific regions between which inductive interaction takes place; and (3) following prolonged exposure to a dissociative procedure, inductive activity can persist in surviving cells, though it is not detectable at any time in the cellular debris or in the medium.

MATERIAL AND METHODS

The dissociation procedure.

The procedure adopted combines physical forces supplied by a powerstat-controlled, 60-cycle vibrator with enzymatic action in a low calcium and magnesium environment at incubator temperatures for 10–12 minutes. The mechanical forces are employed primarily to move the suspending medium as a rapidly swirling thin layer around the walls of a small tube suspended from the vibrator. Under these circumstances the forces exerted on the tissue and cells are assumed to be primarily shearing rather than vibrational, similar to those exerted in drawing tissue in and out of a pipette, but acting continuously, uniformly, and subject to control of intensity. The exact relation between the intensity of the actual disaggregative forces and the intensity of vibration has not been determined, but it probably is not a simple function through the entire available range. For ordinary disaggregation the shaker has been used at the "swirling point", i.e. power is increased to the fairly critical level where the fluid leaves the bottom of the tube and swirls on the walls. Unless otherwise specified below, this procedure may be assumed to have been followed. Above the swirling point, agitation becomes increasingly violent and the probability of cell damage rises.

The disaggregating medium is basically that of Moscona [14] supplemented by pancreatin. The latter was added on finding, in some of the earlier experiments, that with certain batches of dessicated trypsin powder a good deal of gummy material was produced which enmeshed cells and cell clumps and interfered both with complete disaggregation and with effective sedimentation in the centrifuge. This material was found to disappear in 3 per cent pancreatin solution, which by itself was not as effective in dissociating cells as trypsin. The combined enzyme preparations consistently gave good disaggregation without gumminess, and were used routinely in the later stages of these studies.

Details of the procedures are as follows:

Shaker assembly (Fig. 1). —Short lengths of plastic rod (*E*) are fastened to a plexiglas disc (*D*) attached to the free vibrating arm (*B*) of a shaker[1] (*A*) by means of an

[1] Stevens pipette shaker, #8585, W. M. Welch Scientific Co., Chicago 10, Ill., U.S.A.

aluminum connecting rod (*C*). All connections are rendered firm to allow maximum transmission of vibration. Tissue in 0.01 ml of a suitable fluid is placed in glass tubes (O.D. 4 mm, I.D. 2 mm, length 19.3 mm) sealed at one end (*G*). The length of the tube is critical; the sealed end of the tube is required to be smooth, symmetrical and either flat or slightly concave. The tubes are attached to the assembly by means of a sleeve-type, 10 ml serum bottle cap (*F*); the tube fits snugly into the lower section of the flexible connector while the sleeve of the cap fits tightly around the rods projecting from the plexiglas disc. Both tubes and rods are ordinarily inserted as far as possible, each making contact with the center shelf of the cap. The flexible connection allows the tube to shake with the connector as pivot point. While the tubes are carefully selected to be of identical lengths, minor compensations can be made by varying the degree of insertion of the tube in the connector. The distance from the bottom of the tube to the center of the serum bottle cap is one-half the length of the standing wave induced by the shaker, so that the lower end has maximum displacement. The amplitude of vibration is adjusted by a powerstat. With increasing amplitude, motion of the fluid is gradually increased to the critical swirling point, then is more and more violently agitated.

Dissociation medium.—The utility of various dissociation media[1] was explored. Crude trypsin, crystalline trypsin, crystalline chymotrypsin and di-sodium versenate all effectively separated the cells [4, 14, 26]; solutions of crystalline carboxypeptidase, hyaluronidase, lysozyme, amylase and lipase were ineffective in disaggregation (cf. [4]). However, crystalline trypsin and chymotrypsin, versenate, and some lots of crude trypsin resulted in the appearance of gummy material [4] which trapped single cells and small cell masses, interfering with the completion of disaggregation as well as with subsequent centrifugal sedimentation of cells. In a comparison of lots of crude trypsin which were or were not gum-producing it was found that non-gum-producing trypsin preparations could actively remove gum resulting from other treatment. The gum-removing activity is heat-labile (70°C) and non-dialyzable, and is not shown by any of the crystalline preparations mentioned above. Crude pancreatin, a dessicate of fresh, activated pancreatic juice, is an effective gum-removing agent, being of the order of ten times as effective as the best crude trypsin preparations. However, it was found to be less active in the cell-separating activity associated with the proteolytic enzymes. The solution finally adopted as a dissociating medium consisted of 3 parts of crude trypsin (Difco 1:250) to 1 part of pancreatin (4 × USP) made up as a 3 per cent solution in tyrodes without calcium and magnesium salts [14].

Production of cell suspensions.—Treatment of tissues at the "swirling point" for 10–12 minutes at 37.5°C uniformly led to a viable suspension almost exclusively composed of single cells (Fig. 5). Examined under phase contrast, 2–6 pairs were seen in each group of 100 cells, while cell clusters of more than two cells were not found. At least 95 per cent of the cells appeared to be healthy by morphological criteria. The shaker assembly also was routinely used to ensure complete mixing of suspensions from two different sources, and to resuspend cells in nutrient medium after centrifugation and removal of the disaggregating fluid.

[1] Crude trypsin: Difco 1:250; all crystalline enzymes: Worthington Biochemical Corp.; di-sodium versenate: Bosworth Chemical Co.; pancreatin and pancreatin 4 × USP: Nutritional Biochemical Corp.; pancreatin 4 × USP also from Viobin Corp.

Dissection of rudiments.—For experiments in which a considerable amount of metanephrogenic mesenchyme was required, 16–20 metanephric rudiments were placed in each of several tubes in crude trypsin at room temperature for 5 minutes. On subsequent swirling for 30 seconds the rudiments regularly broke up into pieces of mesenchyme and structurally intact epithelia. For dissection purposes the epithelia were then picked out, leaving a pure mesenchymal preparation. Incidentally, it was found that dorsal cervical and thoracic regions of 9-day mouse embryos could be dissected en masse to yield individual somites, irregular pieces of spinal cord, cells and clumps of cells, and all of the somites present in the original mass could be recovered.

Tissue Source

Embryonic tissues were obtained from 11-day mouse embryos produced by crosses of BALB/C females with C3H males. Dissections were performed in horse serum–tyrode solution (1:1) and tissues were stored in an atmosphere of 5 per cent CO_2 during the operative period. Metanephrogenic mesenchyme was isolated from individual metanephric rudiments with trypsin by the procedure previously described [7]. Dorsal spinal cord was obtained from the level of somites 2–9 and comprised the region of the cord dorsal to the lateral fissure. Details have been published elsewhere [7].

Culture

The nutrient medium was composed of horse serum, chick embryo juice and tyrode solution (2:1:2) supplemented with penicillin, streptomycin and mycostatin in concentrations recommended by the manufacturers. Plasma clot cluster cultures were made by orienting pieces of metanephrogenic mesenchyme around a central piece of dorsal spinal cord in a clotting mixture of adult chicken plasma and nutrient medium (1:1). Hanging drop cultures were prepared by suspending tissues in drops of nutrient medium from the loosely fitting flat cover of a tissue dish partly filled with tyrode solution to prevent evaporation. All cultures were incubated at 37.5°C in an atmosphere of 5 per cent CO_2 saturated with water vapor. Additional details have been given previously [7].

Reaggregation

Cell suspensions, prepared on the shaker assembly, were centrifuged for 2 minutes at a force of $25 \times g$, the supernatant removed, and the cells resuspended in nutrient medium by means of 30-second swirling on the shaker assembly. Cells were allowed to reaggregate in a millipore filter assembly [8]. A millipore filter disc is mounted on a plastic ring and suspended, filter side down, over the well of a tissue dish. A drop of the cell suspension is placed in the cup of the assembly (Fig. 2). Some filtration occurs and additional drops may be added to the cup as required by removing fluid from the underside of the filter. The drop, when stabilized at the final step, is of constant volume (0.003 ml) so that the concentration of cells becomes a direct function of the total number of cells introduced. The cells remain uniformly dispersed over the total area and reaggregation tends to be somewhat less variable than in a drop on glass (Figs. 3, 4). Additional nutrient medium (0.2 ml) added as a standing

drop below and in contact with the filter, acts both to preserve the volume in the cup and to provide a relatively large nutritional environment for exchange through the filter. It is to be noted again that the assembly is incubated in high humidity which keeps evaporation to a minimum.

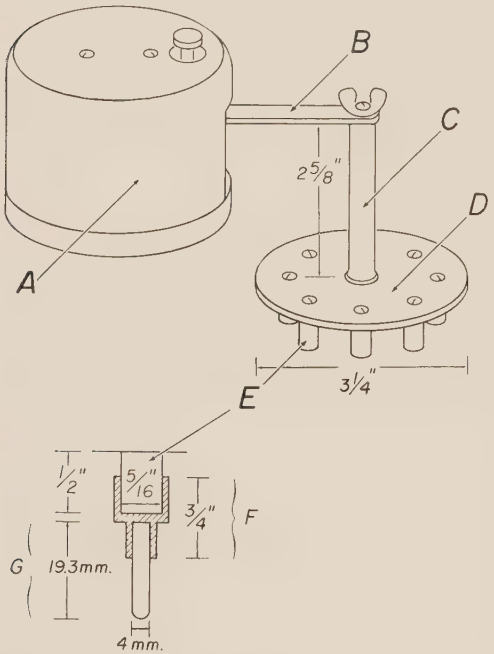


Fig. 1.

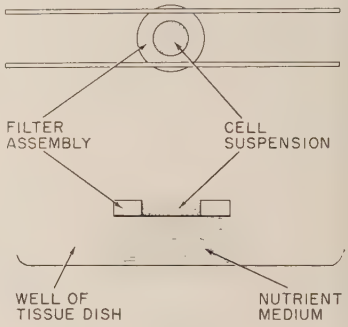


Fig. 2.

Fig. 1.—Shaker assembly. Description in text.

Fig. 2.—Procedure for reaggregation. Description in text.

Histological

Clots were fixed in Zenker-acetic, sectioned at 5 microns, and stained with hematoxylin and eosin. Tissues from hanging drop cultures were first embedded in plasma clots, incubated for 30 minutes, and then handled as indicated. Cell suspensions were fixed for phase contrast study by the addition of approximately one third volume of 40 per cent formol-saline and prepared as wet mounts; coverslips were sealed with a mixture of paraffin and balsam (2:1) to retard evaporation.

RESULTS

Effect of disaggregation on subsequent interactive capacity

Metanephrogenic mesenchyme. Mesenchyme from 32, 46 or 52 kidney rudiments was pooled and disaggregated to cells on the shaker assembly. After centrifugation, removal of the supernatant, and resuspension in nutrient medium, the cells were immediately transferred for reaggregation to the wells

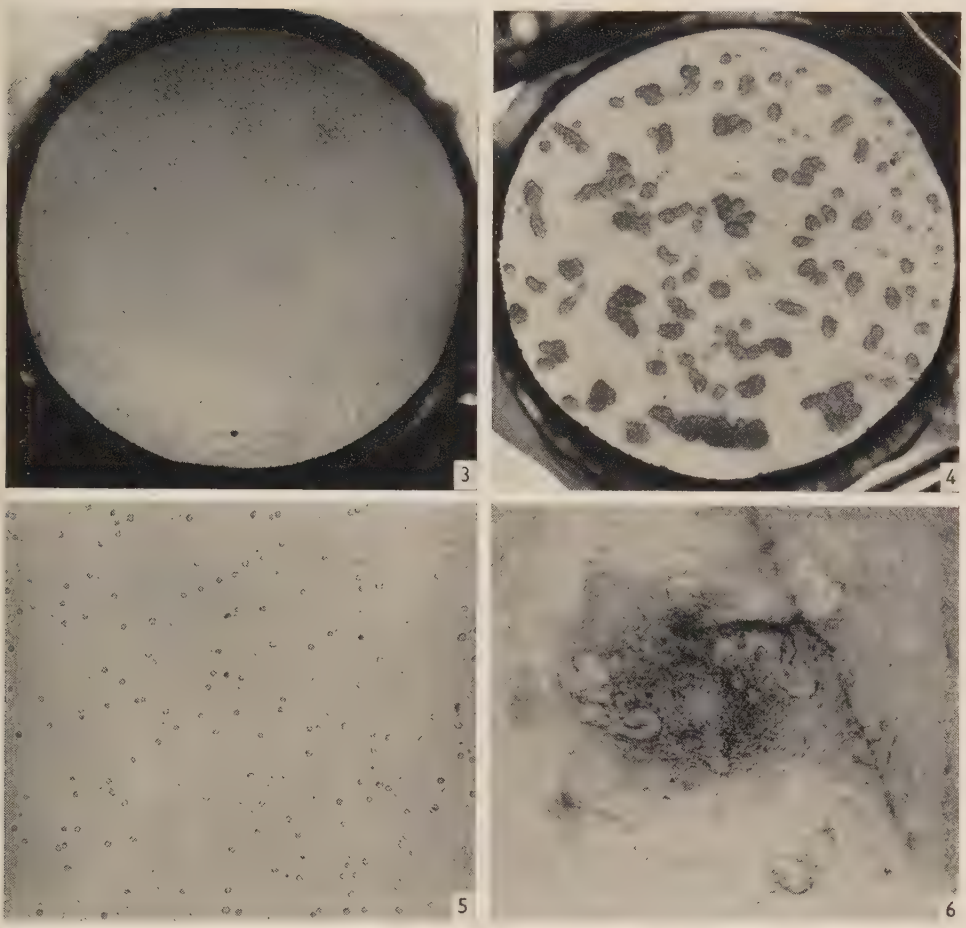


Fig. 3.—Suspension of spinal cord cells on filter (× 24).

Fig. 4.—Overnight reaggregation of suspension shown in Fig. 3 (× 24).

Fig. 5.—Fixed, unstained spinal cord suspension (× 85).

Fig. 6.—Tubule formation by reaggregates of metanephrogenic mesenchyme after combination with dorsal spinal cord and cultivation for 72 hours at the glass-clot interface (× 60).

of two or three filter assemblies, so that the cells from approximately 16 rudiments were concentrated on each filter. The assemblies were incubated overnight. To test for tubule-forming capacity, groups of four reaggregates were clustered around a piece of freshly dissected dorsal spinal cord in a plasma-clot cluster culture. Such reaggregates spread and fused around the spinal cord, producing on the 2nd to 3rd day tubules similar to those seen in

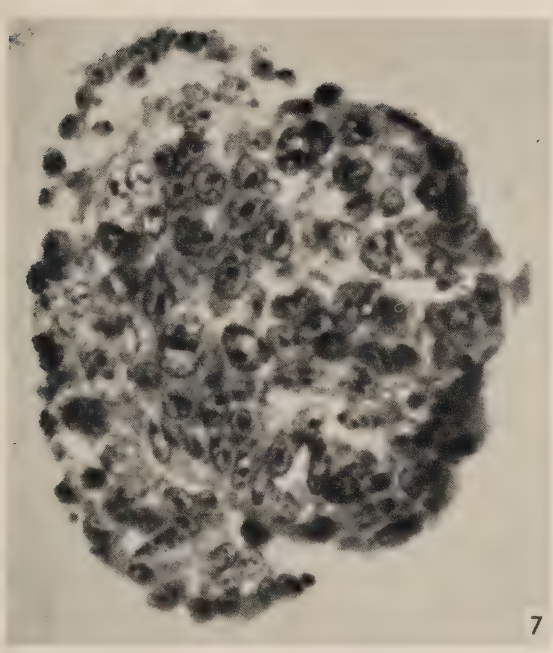
combinations of untreated metanephrogenic mesenchyme and dorsal spinal cord (Fig. 6). Similar cultures of reaggregates in groups of four without spinal cord spread and fused to form a homogeneous thin layer in which tubules or other formed structures were never observed. The experiment was repeated three times. Thirty-eight cluster cultures with dorsal spinal cord all produced tubules, and 19 control cluster cultures failed to do so.

Dorsal spinal cord.—Spinal cord tissue obtained from 3 or 6 embryos was pooled and disaggregated as above. After centrifugation and resuspension in nutrient medium the cells were transferred to filters in such fashion that each filter contained the dorsal cord cells from approximately three embryos. Filters were incubated overnight to permit reaggregation. To test for tubule-inducing capacity, a single reaggregate was placed at the center of a cluster culture of mesenchyme fragments obtained from one freshly dissected metanephric rudiment. In an initial series, the experiment was repeated twice, yielding tubules in 14 out of 18 cultures with dorsal cord reaggregates, and no tubules in 10 cultures of metanephrogenic mesenchyme without dorsal cord. In 4 later experiments, 23 cultures with metanephrogenic mesenchyme and reaggregated dorsal spinal cord were made and all produced tubules.

It is concluded that disaggregation of dorsal spinal cord or of metanephrogenic mesenchyme, when followed by immediate reaggregation, does not destroy the ability to participate in inductive interaction.

Mixed reaggregates.—In one experiment suspensions of metanephrogenic mesenchyme cells and dorsal spinal cord cells were prepared simultaneously in separate tubes as previously indicated, and then combined; in another experiment similarly prepared mesenchyme and spinal cord were disaggregated together in the same tube. After centrifugation and resuspension in nutrient medium the mixed suspensions were placed on filter assemblies and allowed to reaggregate overnight. Twenty-six reaggregates were transferred for further culture at the glass-clot interface; 4 reaggregates were cultured in hanging drops. All 30 cultures developed characteristic metanephric tubules.

Since chick spinal cord from 4-day embryos had previously been shown to be inductively active [7], and the usefulness of chimeric combinations for histological study of mixed cell types had been demonstrated [16], a third experiment was performed in which chick instead of mouse spinal cord was used. Suspensions of chick dorsal spinal cord from 4 embryos, and mouse metanephrogenic mesenchyme from 28 rudiments were prepared. Following disaggregation of both, half the material from each suspension was combined and thoroughly mixed on the shaker assembly. In this instance, the filter assembly was not used for reaggregation; instead, the suspensions were placed



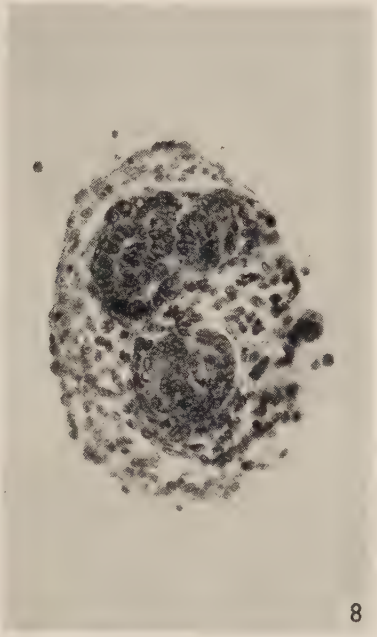


Fig. 7.—18-hour reaggregate of a mixed suspension of mouse metanephrogenic mesenchyme and chick dorsal spinal cord. Chick spinal cord cells to left, mouse metanephrogenic mesenchyme cells to right (× 810).

Fig. 8.—72-hour reaggregate from same suspension as Fig. 7. Typical tubules in mouse metanephrogenic mesenchyme (× 280).

in large drops of nutrient medium in a tissue dish and allowed to reaggregate on glass. A sample of each suspension was fixed for histological study.[1] Three reaggregates from each culture were removed and fixed at 3, 6 and 18 hours. After this time, 30 reaggregates from the spinal cord–mesenchyme mixture and 10 reaggregates from each of the control cultures were transferred to hanging drops and incubated further. By 48 hours all 30 reaggregates from the spinal cord–mesenchyme mixture had produced tubules; no tubules were formed in the control reaggregates.

In histological preparations, the difference between chick and mouse cells was sufficiently characteristic to enable identification of most of the cells in the mixed suspension, but there was sufficient gradation of nuclear size in the sectioned material to prevent positive identification of each individual cell. Nevertheless, areas of characteristic chick and mouse cell concentration were apparent in mixed reaggregates 6 hours after incubation, and by 18

[1] Acetic-formol vapor, Zenker-acetic, hematoxylin [14].

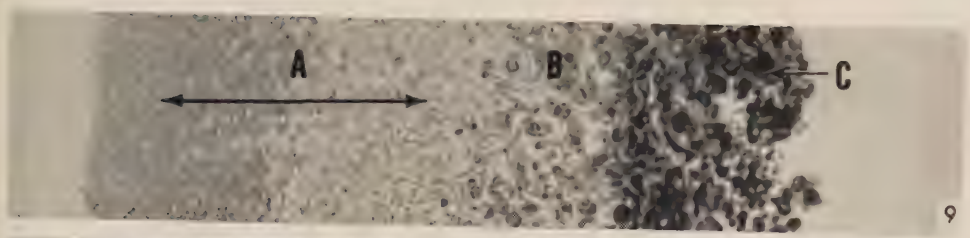
hours grossly observable segregation of cell types had occurred (Fig. 7). Mixed reaggregates cultured 48 hours or more showed the characteristic morphology of early tubule rudiments (Fig. 8).

The experiments demonstrate that in combinations of dorsal spinal cord and metanephrogenic mesenchyme cells, reaggregation leads to an initial mixture of cell types which rapidly segregate into areas of like cell type. Inductive interaction takes place in such mixed reaggregates; whether this begins immediately or only after segregation is not established by these experiments.

Prolonged dissociation of dorsal spinal cord.—A series of experiments was run in an effort to separate activity from living spinal cord cells, by exposing relatively large amounts of dissociated tissue to relatively small volumes of fluid for increasing periods of time. The experiments failed in their original purpose, but are reported for their indication of the difficulty of obtaining cell-free activity, and their demonstration of persistence of inductive activity in surviving cells following prolonged exposure to a dissociative procedure.

Dorsal spinal cord obtained by dissection of 8 embryos in chilled horse serum-tyrode was transferred to disaggregation tubes in cold acid (pH 3.0), near neutral (pH 7.6) or alkaline (pH 9.5) tyrode solution. For treatment near neutrality, tubes were gassed with 5 per cent CO_2. The tubes were attached to the shaker assembly in the cold room at 5°C and were run for intervals up to 10 hours at an intensity of shaking higher than normal and sufficient to cause both separation and gradual destruction of the cells. After treatment, the tubes were centrifuged under refrigeration for 8 minutes at a force of $375 \times g$. The supernatant was removed, neutralized where required with KOH or HCl using phenol red as an indicator, and supplemented with 10 per cent of a 2:1 mixture of horse serum and chick embryo juice with added antibiotics. The sediment was divisible into three layers. The upper (*A*) and lower (*C*) layers were determined microscopically in fixed sections to be acellular. Layer *A* contained a lighter granular and a heavier, stringy region, differing in eosinophilia. Layer *C* contained a gummy mass of vesicles and amorphous material. The central layer (*B*) included nuclei, debris, and a varying number of intact cells depending on the treatment (Figs. 9–12).

Inductive activity of each layer of the various sediments was tested by combining two or more pieces each with metanephrogenic mesenchyme in a standard plasma-clot cluster culture. To estimate viability, pieces from each sediment layer were cultured in isolation in plasma clots to determine their tendency to form outgrowth sheets. Inductive activity of supernatants was tested by culturing metanephrogenic mesenchyme in each as a hanging drop.



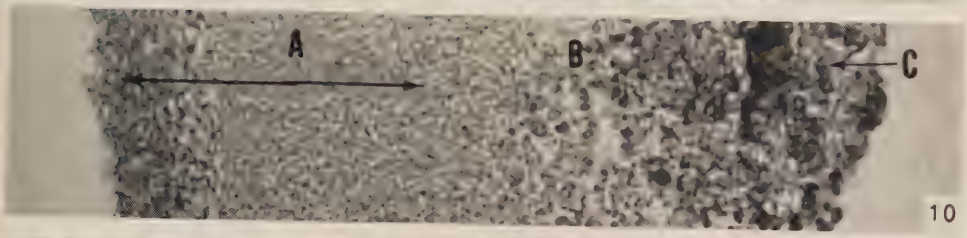


Fig. 9.—Sedimented dorsal spinal cord after dissociation in cold, near neutral tyrode solution for 3 hours. *A*, *B*, and *C* represent characteristic regions described in text (× 365).

Fig. 10.—Sedimented dorsal spinal cord after dissociation in cold, near neutral tyrode solution for 10 hours. Note change in *B* layer.

Fig. 11.—*B* layer of Fig. 9 at higher magnification. Note considerable numbers of reasonably normal nuclei with surrounding cytoplasm (× 1450).

Fig. 12.—*B* layer of Fig. 10 at higher magnification. Note that most nuclei are grossly abnormal. A few reasonably normal nuclei remain, but these appear to be surrounded by little or no cytoplasm (× 1450).

TABLE I. *Tubule-inducing capacity of dorsal spinal cord after prolonged dissociation.*

			Inductive activity				Outgrowth		
pH	Hrs	Supernatants tested	Number active	Sediments tested	B layer number active	A, C layers number active	Sediments tested	B layer number viable	A, C layers number viable
7.6	¼	2	0	—	—	—	—	—	—
3.0	¼	2	0	2	1[a]	0	2[b]	1	0
9.5	¼	1	0	1	1	0	1	1	0
7.6	3–4	2	0	5	5	0	2	2	0
3.0	3–4	2	0	3	0	0	2	0	0
9.5	3–4	3	0	2	0	0	2	0	0
7.6	6–7	2	0	1	0	0	1	0	0
7.6	10	2	0	2	0	0	2	0	0

[a] Very weak response.
[b] Only one piece from each sediment tested; in all other experiments 2 or more pieces were explanted.

The results are summarized in Table I. It will be noted that: (1) at each pH, treatment was continued until activity could no longer be detected in any layer, as judged by the test methods used; (2) despite this, activity was not recorded in the supernatant or in the cell-free sediment layers at any interval of treatment at any pH tested; (3) activity persisted in the middle sediment layer (*B*) as long as viable cells were detectable by culture outgrowth, and disappeared thereafter.

DISCUSSION

It is increasingly recognized that tissue dissociation and reaggregation provide a powerful tool for the study of tissue organization and cellular interaction [6, 11, 12, 17, 21-24]. Greater precision and versatility of technique are, therefore, of increasing importance. The dissociation procedure which has been described in connection with the present studies was developed with these considerations in mind. It provides several advantages: (1) it permits simultaneous and uniform dissociation of a large number of small

tissues, thus providing a pooled cell suspension of satisfactory concentration from embryonic sources of restricted cell number; (2) it is controlled and standardizable, allowing repeated application of the same forces; (3) it can be varied quantitatively, in duration, intensity, incubation temperature, etc.

The element of quantitative control seems particularly important, since with quantitative precision information may be sought on such important questions as the relative effect of different enzyme preparations on particular tissues, the comparative dissociability in the same preparation of different tissues or of the same tissue at different developmental stages, and the relation between dissociability and inductive capacity. Answers to these questions would deepen our understanding of the nature, transformations, and significance of intercellular binding materials in development and pathogenesis [1, 13, 23].

The chief conclusion from the experiments described is that, in the system studied, capacity to participate in induction is not appreciably altered by dissociation followed by early reaggregation. Intermixed suspensions of the interactants segregate and the induction proceeds to the same conclusion as when untreated tissues or reaggregates are placed in contact. If, therefore, any particular arrangement of cells within the interactants is required it is quickly reestablished during aggregation. The conclusion was foreshadowed by earlier results [3, 11, 20]. The present experiments, directed specifically at the question of the effect of disaggregation on inductive interaction, confirm the implications of these earlier investigations. Also, they conform to the previously demonstrated, remarkable tissue specificity of vertebrate reaggregation [15–17, 20, 22, 24] and its apparent independence of species specificity across a wide range [16]. The need for full delineation of the extent and nature of aggregation specificity, taking into account the not fully concordant data on lower forms [2, 18], is clearly indicated. Data on the combining behavior of many different cells and tissues seems fundamental to formulation of a satisfactory theory of aggregation specificity and of tissue organization [23].

In particular, the relation between aggregative behavior and behavior in induction needs further exploration [23]. Townes and Holtfreter [20] have reported sharp differences in aggregative behavior between presumptive neural plate ectoderm prior to induction and neurular ectoderm subsequent to induction. The differences appear in mixed masses with other cells, neurular ectoderm moving inward where pre-induction ectoderm does not. It is not certain that the two kinds of ectoderm would segregate specifically in intermixed masses of the two kinds alone. However, if such changes in aggre-

gation specificity do occur in conjunction with induction the possibility of involvement of a common element in the two processes would be strongly suggested. Such a hypothesis is subject to test with dorsal spinal cord. It would be interesting to know, for example, whether specific aggregation effects can be exerted across a membrane filter and, if so, whether their transmission distance would be the same or different from that of the inductive effect [9].

The results of prolonged dissociation of spinal cord are in accord with the previous finding of a limited transmission distance of induction—in the vicinity of 70–80 microns across a membrane filter [9]. If activity, even under relatively physiological conditions, does not move more than this distance, it is not surprising that it is not easily detected either in supernatants or noncellular debris, and yet persists in the layer containing a significant number of viable cells. The experiment does not indicate that the active material *cannot* be separated from living cells by some suitable procedure. But it suggests that the activity is not *readily* separable—whether due to instability or small quantity—and that it is ordinarily very closely associated with intact living cells. These facts are in conformity with the hypothesis discussed elsewhere [5] that "intercellular" matrix materials are important mediators of inductive processes. It is clear that these same materials likewise are candidates for a role in aggregation.

SUMMARY

1. A standardizable and potentially fully quantifiable procedure is described by which pooled embryonic rudiments of small size in fairly large numbers can be reliably dissociated to varying degrees, including reduction to single-cell suspensions.

2. Using this procedure, it is shown that in the mouse inductive system consisting of dorsal spinal cord and metanephrogenic mesenchyme, dissociation of either interactant does not destroy its ability to participate in induction.

3. Mixed suspensions of the two tissues produce mixed reaggregates which segregate into type specific regions, and induction proceeds to the same conclusion as when untreated tissues or reaggregates are placed in contact.

4. Following prolonged dissociation, inductive activity can persist in surviving cells, though it is not detectable at any time in the cellular debris or in the medium.

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Experimental Cell Research **15**, *398–404 (1958)*

FLUORIDE INHIBITION OF SKELETON FORMATION IN THE SEA URCHIN

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Received December 27, 1957

Herbst [5] first produced abnormalities in sea urchin development by altering the composition of sea water. Since the original studies of Herbst, many investigators have employed artificial sea waters, modified sea waters and inhibitors in attempts to analyze the factors in sea urchin development. These studies have been directed primarily toward an analysis of the grosser aspects of development, such as the establishment of the planes of symmetry [9, 10, 11] or differentiation along the primary egg axis [7]. However, few of these studies have been concerned with the finer aspects of differentiation.

A selective inhibition of calcification in the larvae of the sea urchin *Strongylocentrotus lividus* was demonstrated by Bohn and Drzwina [2]. This inhibition was accomplished by growing larvae in sea water in which a strip of metallic silver had been placed for one minute. They also found that tin enhanced skeleton development. The extent and mode of inhibition were not discussed in their paper. In an abstract which appeared while this manuscript was in preparation, Greenberg and Greenberg [3] have reported skeleton inhibition by fluoride ion in the pluteus of *Arbacia punctulata*.

The embryonic origin of the sea urchin skeleton has been investigated [6] but very little appears to have been done in studying the actual process of calcification and skeleton formation in the sea urchin pluteus. It is the purpose of this paper to confirm and describe in more detail the apparently specific inhibition of skeleton formation in the pluteus of the sea urchin, by fluoride ions.

MATERIALS AND METHODS

When crystalline sodium fluoride is added to sea water, a local precipitate of calcium fluoride is immediately formed which is extremely difficult to redissolve. In order to avoid this local precipitation, a 0.5 *M* stock solution of sodium fluoride was prepared in distilled water. Varying amounts of this stock solution were added

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to sea water, under constant agitation, to give a series of test solutions of the following concentrations: $10^{-3}M$, $2 \times 10^{-3}M$, $5 \times 10^{-3}M$, $10^{-2}M$, $1.5 \times 10^{-2}M$ and $2 \times 10^{-2}M$ sodium fluoride. It was found that in this way the maximum amount of sodium fluoride could be put into solution without precipitation, within the limit of the solubility product of calcium fluoride. All other solutions used were made the same way. Larvae from the sea urchins *Lytechinus variegatus* and *Arbacia punctulata* were used. Observations were made with the dissecting microscope at 54 × magnification, unless otherwise stated.

RESULTS

When reared in NaF of appropriate concentration (5×10^{-3} *M*), the eggs developed normally through the gastrula stage. These treated eggs cleaved normally, produced micromeres which migrated into the blastocoel and finally became swimming gastrulae that were indistinguishable from the sea water controls. However, after about 22 hours (at 23°C) of development, when the control larvae were beginning to form arms, the treated larvae departed from the normal developmental pattern. The skeleton of the normal *Lytechinus* pluteus is easily visible under the dissecting microscope and the first outward sign of its formation may be seen in the protrusion and elongation of arms, induced by the skeletal cells [5]. In the NaF treated embryos, no skeleton formed. The prospective position of the arms was made apparent by the appearance of protuberances which were similar to the controls at the same stage. However, these swellings never elongated. The larvae were pluteus-like except for the absence of arms. The gut was complete and the general shape and movement normal. As development proceeded, the body became larger and rounder than normal, until at about seven days, the pluteus was little more than a large ball with the gut composing a small fraction of the total volume of the larva. These NaF treated larvae lived for a considerably longer time than the controls, some living for as long as two weeks without feeding. The controls seldom survived for longer than five days.

The formation of the skeleton could be inhibited by placing the larvae in the NaF solution at any stage of development up to the gastrula stage and partially inhibited even in the early gastrula. Once the skeleton had formed, however, there was no effect upon the addition of fluoride. Table I lists the dilutions of sodium fluoride tested, shows the skeletal effect of each solution and indicates the relative toxicity and amount of calcium fluoride precipitate formed in each case. The optimum concentration for skeleton inhibition was about 5×10^{-3} *M* sodium fluoride.

TABLE I. *Effect of varying concentrations of NaF on the skeleton of* Lytechinus variegatus *plutei.*

NaF conc. *M*	Skeletal effect	Toxicity	Calcium fluoride precipitate
	Inhibition		
10^{-3}	none	—	—
2×10^{-3}	slight	—	—
5×10^{-3}	complete	—	—
10^{-2}	complete	—	trace
1.5×10^{-2}	complete	sl. toxic	much
2×10^{-2}	—	toxic	much

Reversal of Inhibition

The inhibition by fluoride was reversible. If skeletonless plutei were removed from the fluoride solution, washed in fresh sea water and allowed to develop in sea water, evidence of skeleton formation could be seen within 10–12 hours. This reversal was most effective in young plutei, the older and more abnormal plutei showed correspondingly less skeleton with a greater degree of abnormality of structure. About 5 per cent of the plutei resulting from this treatment of young skeletonless plutei were normal in appearance, the rest showed varying degrees of abnormality. In some, the skeleton was complete but the arms project from the body at atypical angles. In others, the skeleton was incomplete, only one arm or even no arms formed and the skeleton was confined to short spicules which did not grow enough to induce arms. In many of these larvae, the elongating spicules did not project into prospective arm tissue, so that instead of being associated with an arm, the skeleton merely projected through the body wall of the larva. This is in agreement with the observation that the position of the arm evaginations is determined early, though arms will not begin to grow out without the skeleton [5].

This reversal was apparently not simply a dilution effect. If skeletonless plutei were washed in isotonic NaCl and allowed to stand in this solution, they soon died. However, control plutei would survive in solutions of sea water diluted up to 50 per cent with isotonic NaCl. Dilution of fluoride–sea water to this value with isotonic NaCl (pH 8.2) failed to reverse skeleton inhibition. However, replacement of only half of the fluoride sea water

solution with sea water did reverse skeleton inhibition, although this treatment was not as effective as a complete change to sea water. These observations suggested that one or more specific ions in sea water was involved in the reversal of inhibition.

Effect of Mg and Ca Ions

Since fluoride is known to inhibit calcification by blocking phosphate liberation through the formation of a Mg-fluorophosphate complex [4], the effect of Mg ions on skeletonless larvae was studied.

The average sea water contains slightly under 5 g/l of magnesium chloride (5.2×10^{-2} *M*) and about 1 g/l (10^{-2} *M*) of calcium chloride [1]. The addition of magnesium chloride and/or calcium chloride in a series of concentrations up to double these amounts did not reverse the inhibition of skeleton formation caused by as low a concentration of NaF in sea water as would completely inhibit calcification, namely a 5×10^{-3} *M* solution. Phosphate ions, in the form of potassium phosphate or sodium glycerophosphate, were also found to have no effect on the inhibition. The only successful reversal of inhibition was accomplished by replacing at least one half of the 5×10^{-3} *M* NaF solution in sea water with fresh sea water. The higher the NaF concentration, the more sea water must be added to effect reversal.

Effect of pH

The normal pH of the sea water used in these experiments was 8.2. Upon investigation, it was found that raising the pH of the sea water to 9.0 ± 0.3, by adding 0.1 *N* NaOH, prevented the fluoride inhibition of skeleton formation. Increases in pH above 9.3 resulted in abnormal development, and 9.5 was toxic. By lowering the pH with 0.1 *N* HCl to 6.9 ± 0.3, skeleton inhibition was also prevented. Decreases in pH below 6.6 caused abnormal development and pH 6.0 was toxic. Apparently, the influence of fluoride on calcification was pH dependent and the pH range for effective inhibition was about 7.2 to 8.7. The pH of all other solutions used in these experiments was kept at about 8.2.

Effect of Other Compounds

A series of compounds including chelating agents, glycolytic inhibitors, heavy metals, and other inhibitors were also tested for their effect on skeleton formation. Solutions of alanine, glycine, acetylacetone, oxylate and citrate were studied and these chelating agents were found to have no effect on

skeleton formation, with the possible exception of citrate, which appeared to have some effect at toxic levels. This failure of most chelating agents to interfere with normal development is in agreement with the observations of Tyler [13]. Monoiodoacetate, dinitrocresol, chloropicrin and iodoacetamide were also studied and found to have no effect. The effect of silver ions on skeleton formation was studied, using the technique of Bohn [2] except that in this case, instead of a bar of silver metal, silver wire was used. The silver wire was allowed to stand in 50 ml of sea water for one minute, 15 minutes, 30 minutes and one hour. Solutions containing the silver for one hour were toxic, the others were not toxic and had no effect on skeleton formation. The effect of beryllium ions and other heavy metals such as lead, mercury, copper and arsenic were used and found to have no effect. The same was true of bromide and iodide.

DISCUSSION AND CONCLUSIONS

Fluoride is known as an inhibitor of yeast phosphatase, glucosulfatase, acid phosphatase, hydrogenylase, and enolase [8]. Phlorizin (10^{-2} *M*), iodoacetate (10^{-3} *M*) and fluoride (10^{-4} *M*) are all inhibitors of the glycogenolytic cycle [4] and block *in vitro* calcification when phosphorus is available as inorganic phosphate. Fluoride inhibition of enolase [14] is due to the formation of a Mg-fluorophosphate complex.

Bone calcification is conceived of by Robison [12] as a process by which the enzyme phosphatase liberates inorganic phosphate from phosphoric esters and raises, locally, the concentration of the phosphate ion. The product of the concentrations of the calcium and phosphate ions then exceeds the solubility product of calcium phosphate, which precipitates. Apparently, the mechanism involved in *Lytechinus* skeleton formation is much the same. The introduction of fluoride inactivates the magnesium containing phosphatase, with the inhibition obeying the following equilibrium:

$$\frac{\text{(magnesium fluorophosphate) (magnesium enolase)}}{\text{(magnesium) (magnesium fluorophosphoenolase)}} = \text{K (14).}$$

Of the chelating agents used (alanine, glycine, acetylacetone, oxylate and citrate) apparently none was specific enough or penetrated the egg sufficiently to either remove magnesium from the system or form a Mg-chelate-phosphate complex, with the possible exception of citrate. Citrate appeared to have the same effect as fluoride but was rather toxic at the effective level.

It is of interest to note that although addition of calcium and magnesium ions do not effect reversal of this inhibition, sea water is effective. This might be expected because of the nature of the inhibition equilibrium. The addition of magnesium to the medium without removing the fluoride is not sufficient to overcome the equilibrium without reaching a toxic level of magnesium.

The fact that the fluoride-treated embryos lived for a much longer time than the controls was also of interest. It would seem that the metabolic processes of the fluoride-treated larvae were slowed sufficiently by the fluoride to prolong the life span appreciably. Respiratory studies are planned to investigate this possibility.

The eggs of *Arbacia punctulata* were used in repeating this series of experiments with identical results. Observation of internal structure of early *Arbacia* plutei is considerably more difficult than in *Lytechinus*, because of the concentration of pigments in the *Arbacia* egg, but the shape of these skeletonless larvae is so typical and conspicuous as to make identification unmistakable. Also, later plutei developed to such an extent that the skeleton itself, in the controls, was apparent.

SUMMARY

1. The formation of the skeleton of plutei of the sea urchin *Lytechinus variegatus* and *Arbacia punctulata* was inhibited by a solution of from 1.5×10^{-2} *M* to 2×10^{-3} *M* sodium fluoride in sea water.

2. This inhibition was reversed by the addition of fresh sea water but not by the addition of an isotonic NaCl solution or by the addition of magnesium, calcium or phosphate ions.

3. The inhibition of calcification was pH sensitive, the range for inhibition being about pH 7.2 to 8.7.

4. Chelating agents (alanine, glycine, acetylacetone, oxylate and citrate) with known affinities for calcium and magnesium, were tested with negative results, except for near toxic levels of citrate.

5. Inhibitors of the glycolytic cycle (monoiodoacetate, dinitrocresol, chloropicrin and iodoacetamide) were tested and none inhibited skeleton formation.

6. Beryllium was found to have no effect on this system, although it is known to inhibit calcification.

7. A possible mode of action of fluoride is through the formation of a magnesium-fluorophosphate complex, cutting off the source of inorganic phosphate necessary for calcification.

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NUCLEOTIDE AND NUCLEIC ACID METABOLISM IN DEVELOPING AMPHIBIAN EMBRYOS

II. COMPOSITION AND METABOLIC ACTIVITY OF OVARIAN EGG NUCLEIC ACIDS

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Received January 2, 1958

A PREREQUISITE to the understanding of the role of nucleic acids in amphibian embryonic development is the establishment of the chemical nature of ribo- and deoxyribonucleic acids (RNA and DNA) of the amphibian egg. We therefore undertook a study of the mononucleotide composition of the nucleic acids and their metabolic activity, as measured by the incorporation of ^{32}P-orthophosphate.

The reported presence of DNA in the cytoplasm as well as in the nucleus of the frog egg [5] demanded a comparison of the mononucleotide content and metabolic activities of the DNA's isolated from both sources. Accordingly, we developed a method that independently demonstrates the existence of cytoplasmic DNA in the egg and also yields some information about its metabolic activity in relation to nuclear DNA.

MATERIALS AND METHODS

Procedure for total ovarian and liver nucleic acids

Females of *Rana catesbeiana* received one to seven daily intraperitoneal injections of 1.0 mc of ^{32}P as orthophosphate. When the desired number of injections had been given, the animals were pithed and the ovaries and liver excised immediately. Each organ was homogenized separately in ice-cold 1.0 *M* perchloric acid in a Waring Blendor. The acid-soluble compounds and lipids were extracted and the nucleic acids isolated by extraction with 2 *M* NaCl in a 95°C bath [10]. After dialysis, the nucleic acid fraction was made 0.1 *M* with respect to NaOH and incubated 15–18

[1] Work done while on leave of absence from Southern Illinois University, Carbondale, Illinois, and on an Oak Ridge Institute of Nuclear Studies Research Participantship at Oak Ridge National Laboratory. The preliminary studies that led to this work were supported by a grant from the National Science Foundation.

[2] Operated by Union Carbide Nuclear Company for the U.S. Atomic Energy Commission.

hours at 37°C to hydrolyze the RNA to mononucleotides. The DNA was then recovered by acid precipitation. Inorganic phosphate and a contaminant containing a high content of organic phosphate present in the RNA fraction had to be removed. The nucleotides were adsorbed batchwise on charcoal[1] in an acid medium, thoroughly washed with distilled water, then recovered by elution with hot acetone-ammonium hydroxide solution (94 ml of 40 per cent acetone: 6 ml of concentrated NH_4OH). Although the procedure gave only about 60 per cent recoveries, the percentage yields of each mononucleotide were equal. Samples were evaporated to remove the acetone and ammonia, made 0.05 0.1 *M* with respect to NH_4OH, and the nucleotides were separated by column chromatography [2] by use of a chloride elution system.

Mononucleotides were obtained by hydrolysis of the DNA fraction with crystalline DNAse and then with a snake venom phosphodiesterase preparation [6]. After enzymic hydrolysis, each sample was adjusted to pH 4.5, and placed in a water bath at 90°C for five minutes. This procedure prevented further hydrolysis of the mononucleotides by inactivating small quantities of 5′ nucleotidase apparently still present in the snake venom preparation. The samples were then chilled in an ice bath for half an hour and a precipitate that formed was removed by centrifugation. The precipitate contained substances of high phosphorus content that, by spectral analysis, were not nucleic acids or nucleotides and that interfered with the column separation of the DNA mononucleotides. After the precipitate was removed, the DNA mononucleotides were made 0.05–0.1 *M* with respect to NH_4OH and separated by column chromatography.

The RNA and DNA mononucleotide fractions were evaporated to a convenient volume, the absorption at 250, 260, 280, and 290 mμ was measured in a Beckman spectrophotometer, and an aliquot from each column fraction was counted by use of a thin mica-window Geiger tube. The phosphorus content of each fraction was calculated from the optical density reading at 260 mμ, the appropriate extinction coefficient being used [11].

Procedure for nuclear and cytoplasmic DNA

After the animal had received seven daily injections of 1.0 mc of ^{32}P, the mature ovary was excised and placed in amphibian Ringer's solution. Mature ovarian eggs were selected, placed in calcium-free nuclear medium, and the germinal vesicles of 315 oocytes removed manually [8]. The germinal vesicles and the cytoplasmic material, devoid of the follicular cells and vitelline membranes, were immediately frozen separately. Both fractions were mixed with ovaries from a noninjected female and the nucleic acids and their component mononucleotides were isolated and counted as described. In this way the radioactivities of the mononucleotides reflect the contribution, from DNA and RNA, of the separated cytoplasm and nucleus, whereas the unlabeled ovaries serve as a carrier during the isolation and a marker for the ultraviolet identification of the mononucleotides during chromatography. These data therefore yield information only on total activities associated with the nucleic acids; they do not allow meaningful information on specific activities.

[1] The charcoal used in these experiments was coconut charcoal, 6 14 mesh (Nuchar, Industrial Chemical Sales, New York) activated by boiling in 2 *N* HCl for five minutes and then washing with distilled water until neutral.

TABLE I. *Nucleotide composition of ovarian and liver nucleic acids (ovary, 6 samples; liver, 5 samples).*

Nucleotide	Percentage of nucleotide in Ovarian RNA	Liver RNA	Nucleotide	Percentage of nucleotide in Ovarian DNA	Liver DNA
Cytidylic	24.2±1.6	25.5±1.6	Deoxycytidylic	22.0±1.6	23.2±0.8
Adenylic	25.4±1.4	26.7±0.9	Deoxyadenylic	28.5±1.8	26.8±0.8
Uridylic	16.4±1.6	15.8±2.0	Thymidylic	29.0±2.6	29.5±0.9
Guanylic	34.0±2.8	31.9±2.5	Deoxyguanylic	20.3±0.7	20.5±0.7

RESULTS

Composition of ovarian and liver nucleic acids

RNA.—The composition of the RNA in the ovaries is remarkably constant regardless of whether the ovary contains primarily first, second, or third year egg types. As can be seen from Table I, there is no significant difference between the RNA of the ovary and the RNA of the liver; both show a low uridylic acid content and a high guanylic acid content. As an approximation, in both the ovarian and liver RNA's, C + A (cytidylic and adenylic acids) = U + G (uridylic and guanylic acids).

DNA.—No significant differences in mononucleotide content of DNA in the ovary and liver were found. As shown in Table I, the nucleotide composition of both types of DNA approach the predicted base equalities in that C = G and A = T (thymidylic acid). In addition, the DNA's from frog egg and liver can be described as the high AT type, with a ratio of A + T to G + C of about 1.35 [1]. Eggs from ovaries that contained primarily eggs of the first, second, or third year types had the same relative mononucleotide content of the DNA.

Specific activities of ovarian and liver nucleic acids

RNA.—Table II shows that at no injection level, in the same animal, was the specific activity of the ovarian RNA greater than that of the liver RNA. This was true regardless of the predominant egg type found in the ovary.

The specific activities of the individual mononucleotides were never equal at any injection level tested, which indicates a nonuniform labeling of the RNA in both the ovary and the liver. As an approximation, however, the specific activities of U + G = C + A.

TABLE II. *Specific activities of ovarian and liver nucleic acids.*

No. of daily injections	Predominant egg type	Ribonucleic acid			Deoxyribonucleic acid		
		Ovary [(cts/min)/μg of P]	Liver [(cts/min)/μg of P]	Ratio ovary/liver	Ovary [(cts/min)/μg of P]	Liver [(cts/min)/μg of P]	Ratio ovary/liver
1	First year	1.1	3.2	0.34	12.1	14.4	0.84
2	First year	21.1	83.7	0.25	12.0	11.1	1.08
5	First year	6.2	29.1	0.21	26.5	17.3	1.53
3	Third year	134.1	240.0	0.56	54.2	6.0	9.03
7	Third year	324.5	1680.5	0.19	189.5	18.7	10.11

DNA.—The specific activities of the ovarian and liver DNA's are strikingly different, depending on the type of ovary tested. In Table II it can be seen that the specific activities of DNA in young ovaries (which contain mainly first year eggs) approximate the specific activities of the liver DNA. Specific activities of the DNA in ovaries containing primarily mature eggs are about ten times as great as the specific activities of the corresponding liver DNA.

The specific activities of the mononucleotides from the first year eggs are about equal, indicating a fairly uniform labeling of the DNA molecule; in contrast to a somewhat nonuniform labeling in the mature egg, in which the activity of deoxyguanylic acid is slightly high. The liver mononucleotides show a generally uniform labeling much like that found in first year eggs.

Cytoplasmic and nuclear nucleic acids

RNA.—The distribution and percentage composition of the ^{32}P in the nucleotides isolated from cytoplasmic and nuclear RNA are shown in Table III. On the basis of total counts, activity is much greater in the nuclear RNA than in the cytoplasmic RNA from the same mature eggs. The nuclear RNA differs from the cytoplasmic RNA in that the percentage incorporation of ^{32}P in the uridylic acid is substantially higher in the nuclear RNA than in the cytoplasmic RNA.

DNA.—The data presented in Table III indicate that, on the basis of ^{32}P content, there is indeed DNA in the cytoplasm of mature frog eggs; the extent of ^{32}P incorporation in cytoplasmic DNA is about twice that found in nuclear DNA. The distribution of the counts within the mononucleotides of cytoplasmic DNA parallels that of the whole mature egg. The nuclear DNA differs from this pattern in that the amount of radioactivity in the deoxyguanylic acid phosphorus seems disproportionately high.

TABLE III. *Distribution of ^{32}P in the nucleotides of cytoplasmic and nuclear nucleic acids.*

otide	Cytoplasmic RNA Total[a] counts	Cytoplasmic RNA Percentage of tot. cts.	Nuclear RNA Total[a] counts	Nuclear RNA Percentage of tot. cts.	Nucleotide	Cytoplasmic DNA Total[a] counts	Cytoplasmic DNA Percentage of tot. cts.	Nuclear DNA Total[a] counts	Nuclear DNA Percentage of tot. cts.
ylic	50.0	19.3	93.4	11.6	Deoxycytidylic	97.2	15.7	40.0	14.2
ylic	91.0	35.1	195.2	24.2	Deoxyadenylic	191.5	31.0	78.0	27.8
lic	32.0	12.3	344.0	42.7	Thymidylic	195.0	31.6	76.6	27.2
ylic	86.6	33.3	174.0	21.5	Deoxyguanylic	133.0	21.7	86.5	30.8
Total	259.6		806.6		Total	616.7		281.1	

[a] Counts per minute per total nucleotide.

DISCUSSION

The mononucleotide composition of the RNA's of amphibian eggs and liver is similar to that of RNA's from other sources in that both have a high guanylic acid and a low uridylic acid content and, in addition, show a tendency for guanylic plus uridylic acid to equal cytidylic plus adenylic acids [7]. No differences were observed in composition of first and third year eggs.

The mononucleotide content of the total DNA's of the ovarian eggs and liver show no striking deviations from the generalization that A = T and C = G [1]. It should be emphasized, however, that the nucleotide composition of the DNA of amphibian eggs represents *total* DNA, i.e., nuclear *plus* cytoplasmic. If either type is present in relatively small amount, its composition may deviate from this generalization. No differences in nucleotide composition of total DNA were found between first and third year eggs.

In our experiments, the specific activities of the ovarian RNA's were always lower than those of the liver RNA's, regardless of egg type or ^{32}P injection levels. This may be contrasted with the specific activities of the DNA's from ovary and liver. As was pointed out, the specific activity of the DNA from first year eggs approximates that of the liver DNA. On the other hand, the specific activity of the total DNA from third year eggs is much greater than that of the corresponding liver DNA. This increased activity of DNA in mature eggs may be a reflection of an increased rate of DNA synthesis. Grant [4] observed that during the third year of development, amphibian eggs show the greatest growth rate and highest total nucleic acid content.

The relative activities of the nuclear and cytoplasmic DNA's also bear on this problem. The great amount of radioactivity in the DNA of the nucleus compared to that of the cytoplasm may be explained in one of two ways: (*a*) the nuclear DNA exhibits a greater metabolic activity than the cytoplasmic DNA, or (*b*) there is in fact only two or three times as much cytoplasmic DNA as nuclear DNA, and consequently the total counts are strictly proportional to the relative amounts of each type. The second alternative is doubtful on the basis of the microbiological assay work of Hoff-Jørgensen and Zeuthen [5], who found, depending on the species of frog egg, about 10–30 times as much DNA in the cytoplasm as in the nucleus. In addition, England and Mayer [3], by mass isolation of frog ova nuclei and comparison with spermatozoan and liver nuclei, find a DNA quantity in ovarian egg nuclei equivalent only to the diploid amount. Accordingly, if the DNA content of the nucleus is maintained at an essentially constant level, the relatively high uptake of ^{32}P into nuclear DNA may indicate some sort of "turnover" of nuclear DNA. Although this "turnover" may be simply a reflection of concomitant synthesis and degradation of nuclear DNA, we may suggest that the increased radioactivity of nuclear DNA over cytoplasmic DNA is related to increased synthesis of DNA in the nucleus, which in turn might be used for storage in the cytoplasm. The fact that only in the ovary containing predominantly mature eggs does the specific activity of the egg DNA surpass that of the liver DNA may indicate that the "synthesis of storage" of DNA becomes most active in the mature egg. The increased radioactivity of nuclear DNA relative to its content indicates that the frog ovarian egg is an example of active metabolism of DNA in a nondividing nucleus.

An almost identical situation exists with respect to cytoplasmic and nuclear RNA; on the basis of total counts, the nuclear RNA is about 3.5 times as active as cytoplasmic. In this case also, the results probably cannot be explained on the basis of respective RNA content. The high activity of the nuclear RNA is not unique to frog eggs because nuclear RNA of other tissues has been reported to show a greater metabolic activity than cytoplasmic RNA [9].

SUMMARY

A comparison is made of the nucleotide compositions and specific activities of nucleic acids of the ovarian egg and liver of the same animal (*Rana catesbeiana*) injected with ^{32}P-orthophosphate. The nucleotide compositions of the egg RNA and DNA are quite similar to those of the respective nucleic acids of the liver and do not vary significantly with age of the egg. At no time

is the specific activity of ovarian RNA greater than that of liver RNA, but in mature ovarian eggs the specific activity of the DNA is much greater than the liver DNA.

We confirmed the presence of DNA in the cytoplasm of mature ovarian eggs by combining microdissection with radioactivity techniques. After daily injections of ^{32}P-orthophosphate over a period of seven days, cytoplasmic DNA contains about twice the radioactivity of nuclear DNA, whereas the amount of radioactivity in cytoplasmic RNA is only one-third that of nuclear RNA.

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Experimental Cell Research **15**, *412–453 (1958)*

BRIEF NOTES

THE METACHROMASIA OF SODIUM γ-POLY-GLUTAMATE AND PEPSIN[1]

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Received May 28, 1958

Two of the three major groups of naturally occurring macromolecular structures have been shown to be chromotropes [5] and hence capable of producing a distinctive spectral pattern in certain basic (metachromatic [5]) dyes. The mucopolysaccharides have long been known to behave in this manner and more recently nucleic acids [8] have been shown to react similarly. The present report demonstrates that the third major class of compounds, i.e., polypeptides and proteins, may display similar activity.

Two polypeptides were investigated which were composed of different amounts of D and L isomers of glutamic acid. A γ-poly-D-glutamate (AGP) was obtained from spores of *B. anthracis*[3] and a γ-poly-D-L-glutamate (47 per cent D–53 per cent L) (SGP) was isolated from growing cultures of *B. subtilis*. SGP produced typical metachromatic spectral changes in toluidine blue (TB), crystal violet and methylene blue whereas AGP was effective only with TB. Moreover, 2.5 times more AGP than SGP was required to produce an equivalent spectral change in TB. Since the AGP preparation had the higher molecular weight [4], the greater efficacy of SGP[4] as a chromotrope appears to be related to its content of the L optical isomer. AGP behaved as a typical chromotrope in that the change it induced in the absorption spectrum of TB was reduced or annulled as (1) the ionic strength (acetate buffer) increased, (2) the ionization of carboxyl group was repressed by decreasing the pH, (3) the temperature was raised, (4) ethyl alcohol replaced water. Ethyl alcohol succeeded in abolishing metachromasia between 8 and 25 per cent (v/v).

The pepsin was a 2X-recrystallized preparation.[5] Fig. 1 shows the metachromatic behavior of TB on the addition of pepsin. The curves are quite similar in appearance

[1] Aided in part by grant No. RG-4690 from the National Institutes of Health and by an Institutional Grant from the American Cancer Society.

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[3] We are indebted to Dr. C. B. Thorne of Camp Detrick for these preparations. The physicochemical properties of the *B. anthracis* sample (# 50) has been reported on in detail elsewhere [4].

[4] This preparation (# 100) consisted of 47 per cent D–53 per cent L glutamic acid. A 0.19 per cent solution in 0.15 *N* NaCl had a sedimentation velocity constant of 1.76 S at 20°C.

[5] Pepsin was obtained from the Worthington Biochemical Corp., Freehold, New Jersey.

to the polypeptide curves except for the fact that the 550 mμ band in the latter curves crosses the 630 and 590 mμ bands at their intersection point and lies above them beyond this point. In dilute HCl (pH ~3.5) the metachromatic reaction was no longer observable.

From the concentrations of reactants listed in the legend to Fig. 1 it is apparent that the molarity of TB considerably exceeds that of pepsin. However, if we consider that pepsin possesses 38 carboxyl groups [7] (out of 327 residues) then the concentration of negatively charged groups is increased by this factor if all these groups are ionized. On this basis there are as many carboxyl groups as TB molecules when 0.15 cc of pepsin has been added. The full metachromatic effect is observed therefore when there are about as many dye molecules as carboxyl groups.

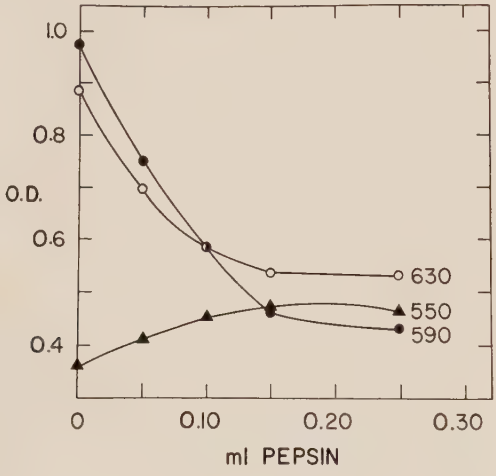

Fig. 1.—Effect of pepsin on optical density of 3 ml of toluidine blue at 25°C. Toluidine blue = 39 μM. Pepsin = 10.5 μM (0.38 per cent) in 0.002 M phosphate buffer, pH 6.0.

When pepsin was denatured either thermally (>70°C) or by alkali (>pH 9), the spectral curves obtained with these preparations were similar to those illustrated in Fig. 1 with native pepsin. However, the decrease in optical density (of the 630 and 590 mμ bands) was 15 20 per cent greater with denatured pepsin. This enhancement in reactivity may be correlated with the increase in the number of charged carboxyl groups that occurs at pH~6 on denaturation [1]. It appears therefore, that the molecular form of the chromotrope does not appreciably influence the metachromatic response since the shape (and electrostatic free energy) of native pepsin is profoundly modified when denatured [2].

Since about 11.5 per cent of the amino acid residues in pepsin are negatively charged the average distance between charges along the extended polypeptide chain is about 3.64 Å/0.115 -32 Å. The negative charge density of denatured pepsin is therefore smaller than most other polyanions exhibiting metachromasia [6].

The interaction of TB with pepsin may also be discerned by its ability to affect the rate of pepsin inactivation. It can be seen from Fig. 2 that significant stimulation was encountered when the molar concentration of dye was similar to that of pepsin. It is of interest to note that a plot of the logarithm of pepsin activity was not linear

with time. The form of the inactivation curve resembles that of an autocatalytic reaction. Of a large group of denaturing agents [3] (urea, guanidine, ethyl alcohol, trimethyl dodecyl ammonium chloride) investigated with pepsin only TB did not decay according to first order kinetics.

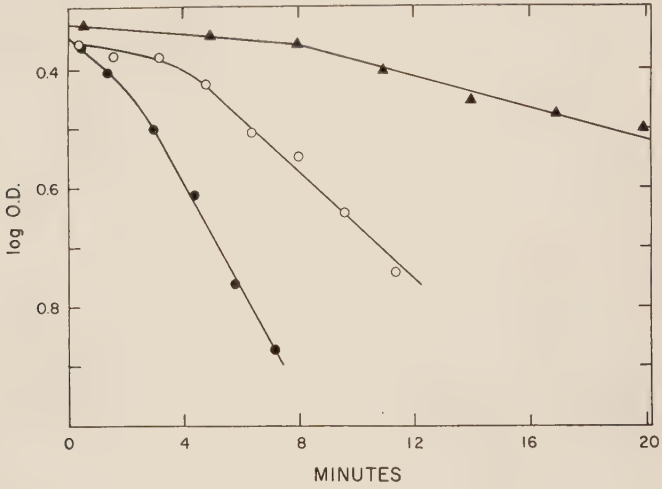

Fig. 2.—Rate of pepsin inactivation. Pepsin = 28 μM. Toluidine blue: △, ○, • = 50, 100 and 200 μM respectively. pH = 7.3; $KNO_3 = 2.5 \times 10^{-3}$ M; T = 28°C. Hemoglobin assay was used for pepsin activity.

Since all three kinds of charged macromolecules normally found in tissues have now been shown capable of inducing metachromasia it is incumbent in any investigation employing this staining reaction to consider the possibility of all three types of structures as being chromotropically active.

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TWO CHEMICALS PROMOTING CELL DIVISION IN THE YOSHIDA SARCOMA CELLS

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Received June 4, 1958

Two chemicals were found by the writer to act as mitotic stimulants on animal cells. One is kinetin and the other is Na-glucuronate.

Kinetin (6-furfurylaminopurine) was isolated by Miller *et al.* from deoxyribonucleic acid from herring sperms as a stimulant on plant cell mitosis [1, 2, 4, 7]. This author investigated the effect of kinetin on the mitotic activity of Yoshida sarcoma cells transplanted to the Wister strain of rat [5], though negative results have been reported by Lettré *et al.* [3]. Injecting the solution of kinetin into the abdomen, a significant increase in the frequency of mitotic figures was found in the tumor cells after 120 hours (significant at the 1 per cent level). Thus I found a case in which kinetin can induce a high rate of multiplication in animal cells as well as in plant cells.

Na-glucuronate is known as a growth promoting substance in animals. Its effect on the division of animal cells, however, was not reported. The present investigation deals with the effect of Na-glucuronate on the mitotic activity of Yoshida sarcoma cells transplanted to the Wistar strain of rat. Na-glucuronate was injected into the abdomen or into the muscle tissue of rat (2 months old) immediately after transplantation of sarcoma cells. Between 2 and 7 days after transplantation, the error variation in the frequency of mitotic figures is very low, and it was convenient to use this period.

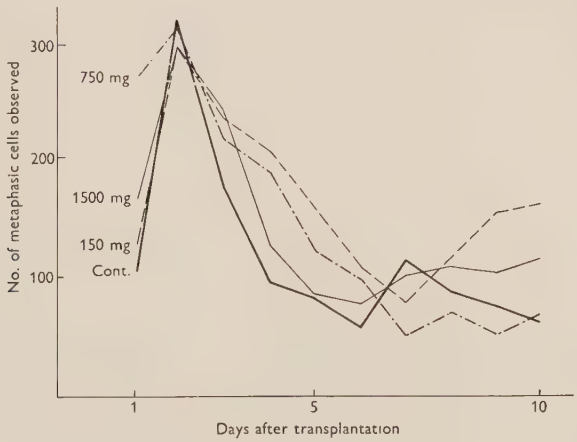

Fig. 1.—Daily change in frequency of metaphasic cells (pathological figures excluded) after injecting Na-glucuronate into the abdomen of rat, 10,000 cells being observed per day.

When a solution of Na-glucuronate was injected into the abdomen of rat, a significant increase in the frequency of mitotic figures was found in the tumor cells after 24 (significant at the 5 per cent level) and 96 (significant at the 1 per cent level) hours as shown in Fig. 1, Tables I and II. But when injected into the muscle tissue,

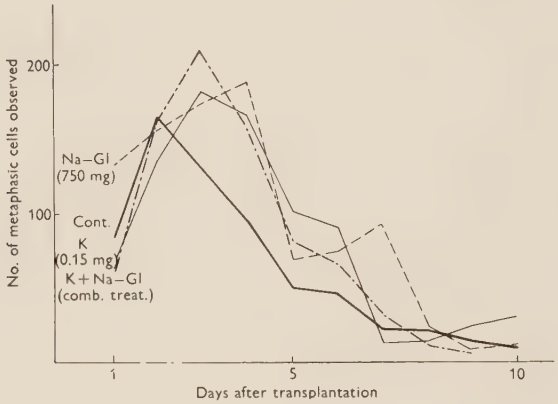

Fig. 2.—Daily change in frequency of metaphasic cells (pathological figures excluded) after injecting kinetin, Na-glucuronate and both chemicals combined into the abdomen of rat, 10,000 cells being observed per day.

TABLE I. *Frequency of mitotic figures obtained by injection of Na-glucuronate.*

Preparation: Aceto-orcein staining by squash technique. The percentage of dividing cells was determined by observation of 10,000 cells. No dividing cell was found in the ascites if Na-glucuronate only was injected and the sarcoma had not been transplanted.

Time after inject. (hr.)	Control (%)	Na-glucuronate 150 mg (%)	Na-glucuronate 750 mg (%)	Na-glucuronate 1500 mg (%)
24	0.71	1.14	0.75	1.50
	1.08	1.12	3.16	1.22
	0.94			
72	1.64	2.52	8.72	8.21
	1.72	2.06	10.37	9.39
	1.78			
96	1.00	2.04	1.82	1.06
	0.94	2.04	1.88	1.44
	0.85			
120	0.62	1.48	1.42	0.42
	1.32	1.70	0.92	1.24
	0.78			

TABLE II. *Analysis of the frequency of dividing cells.*

Standard error: 24 hr. control, 0.137; after treatment, 0.172; 96 hr. control, 0.114; after treatment, 0.142.

Source	Degree of freedom	Mean of square 24 hr.	72 hr.	96 hr.	120 hr.
Treatment	3	6.0[a]	1.43	3.83[b]	2.14
Error	5	0.588	2.718	0.004	1.356

[a] Significant at the 5 % level.
[b] Significant at the 1 % level.

TABLE III. *Frequency of mitotic figures obtained by combined (III) and separate treatments (I, II) with the most effective dose.*

Time after injection (hr.)	Separate treatment Kinetin (I) (0.15 mg) (%)	Na-gl. (II) (750 mg) (%)	Combined treatment Kinetin + Na-gl. (III) (0.15 mg + 750 mg) (%)
72	1.81	1.29	2.22
	1.67	2.23	2.00

TABLE IV. *Analysis of the frequency of division cells at the combined (III) and separate (I, II) treatment.*

Source	Degree of freedom	Mean of square (III) and (I)	(III) and (II)
Comb. treat.	1	0.15	0.40
Error	2	0.13	0.70

no significant increase was found even when the most effective dose (750 mg per 100 g body weight of rat) when injected into the abdomen was used. This suggests that the effect of Na-glucuronate on sarcoma cells may be direct (preliminary paper in Japanese [6]).

Further, in order to examine the relation between kinetin and Na-glucuronate in the promotion of cell division, the effect of combined treatments of these two chemicals was investigated using the same method as in the above experiments under the most

effective dose (kinetin, 0.15 mg per 100 g body weight; Na-glucuronate, 750 mg per 100 g body weight) as shown in Fig. 2.

At a combined treatment, the promoting effect of Na-glucuronate on sarcoma cell division was not investigated 24 hours after transplantation. After 72 hours, no statistically significant difference could be found between separate and combined treatments as shown in Tables III and IV.

This result indicates that (1) the effect of Na-glucuronate which appears 24 hours after transplantation of sarcoma cells is due to a different mechanism from that which acts after 96 hours; (2) the two chemicals do not help each other in promoting cell divisions in the sarcoma 48 hours and after transplantation of sarcoma cells.

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ISOLATION OF CYTOPLASMIC STRUCTURES FROM YEAST

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Received June 16, 1958

Since the early observations of Guillermond [10] on yeast and fungi, cytological and cytochemical methods suggested the existence in these organisms of cytoplasmic granules resembling the mitochondria of higher plants and animals [4, 12, 20].

More recently, electron micrographs of ultrathin sections of *Saccharomyces cerevisiae* and *Candida albicans* revealed the existence in these cells of ellipsoidal granules with the characteristic internal structure (cristae) of the classical mitochondria ([1] and more clearly Alvarez [3]). The physiological meaning of these structures was suggested by their behavior toward the oxidoreduction dyes which were used for their identification.

In 1943, Chantrenne [5] demonstrated the presence of respiratory enzymes in yeast granules in cell-free extracts. Successively, Slonimski [16], Linnane and Still

[13] and Vanderwinkel *et al.* [17] brought forward evidence showing the localization of the electron-transferring system and the tricarboxylic acid cycle in these structures. Other biochemical activities, such as the synthesis of lipids [2, 6, 11], have also been obtained with such granules.

Few biochemical activities have been analysed with a morphologically well-defined material. This is due to the difficulty of obtaining biochemically active preparations in the cell-free state.

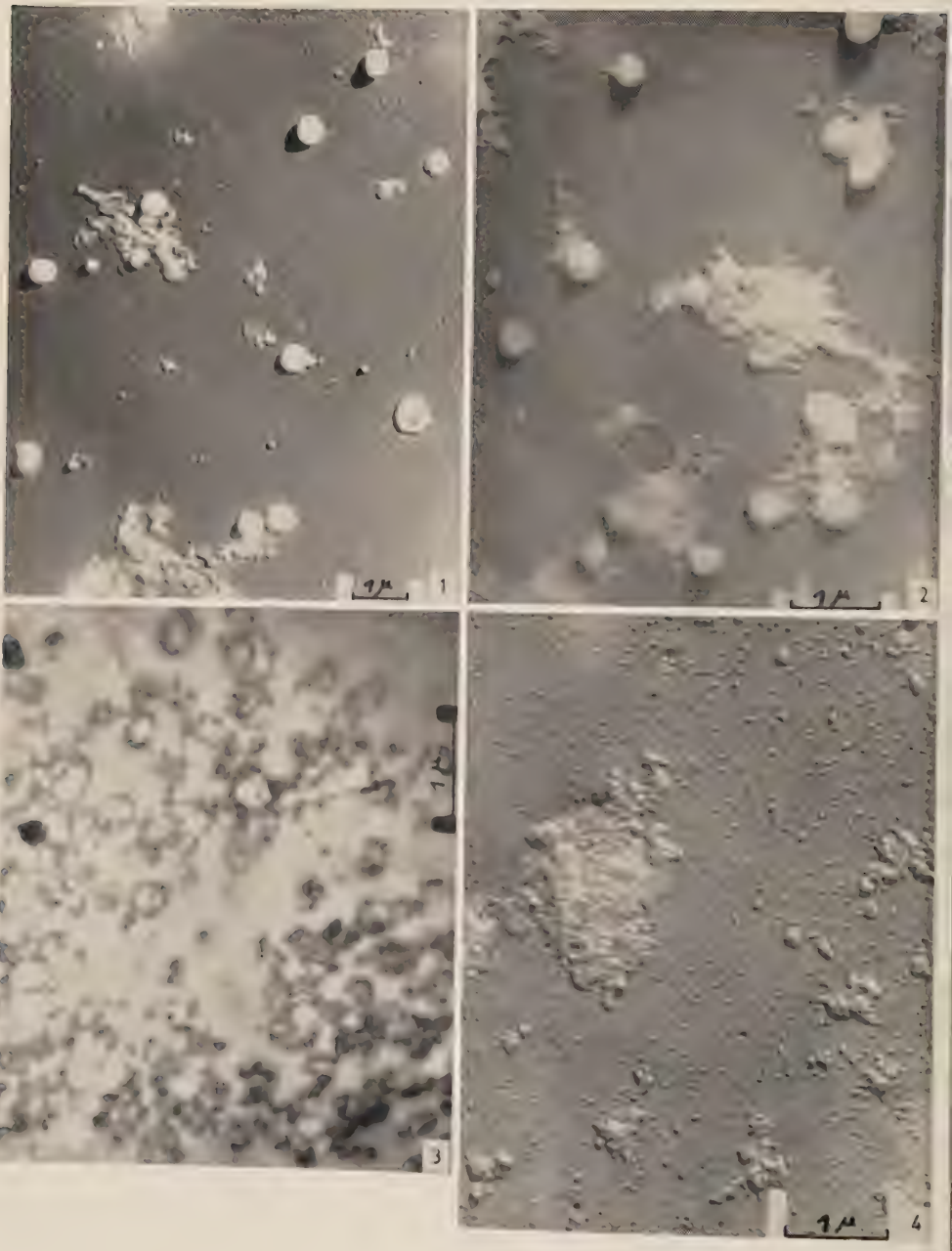
Recently we have reported [17] that cytoplasmic particles, able to oxidize acetate in the presence of catalytic amounts of any of the Krebs-cycle intermediates, could be obtained by ultrasonic disruption of yeast cells under proper conditions. These results suggest that the mixture of granules would contain the respiratory particles in their native state.

It is the object of this note to show the existence of well-defined mitochondria (0.4–0.5 μ) in such preparations. Both shadowed intact mitochondria (Figs. 1 and 2) as well as sections (Fig. 3) are given. Other smaller particles of 25 to 30 mμ diameter are also reported (Fig. 4) in confirmation of Fu-Chuan Chao and Schachman [8].

The preparation is made by the following procedure. 10 g of baker's yeast are suspended in 15 ml of a solution containing: triethanolamine (pH, 7.3), 0.05 *M*; $CaCl_2$, 10^{-2} *M*; $MgCl_2$, 10^{-3} *M* and lactose, 0.5 *M*. The suspension is treated for 5 minutes in a Raytheon (10 Kcycles) oscillator. Intact cells and cell membranes are sedimented into two layers at 3000 *g* for 5 minutes. By centrifugation between 5000 and 15,000 *g*, a sediment of mitochondria (*M*) is obtained. Similar centrifugation between 20,000 and 100,000 *g* for 1 hour gives the sediment (*m*) of small granules. All the operations are made in the cold, resuspensions are obtained with a glass pestle adjusted to the centrifuge tube. The sediment, *M* or *m*, is suspended in a few ml of a buffered hypertonic solution of osmic acid 1 per cent [15]. After 15 minutes at room temperature, the material is centrifuged at 25,000 *g* for few minutes and washed in a solution containing triethanolamine (pH, 7.3), 0.05 *M*; $CaCl_2$, 10^{-2} *M*; $MgCl_2$, 10^{-3} *M* and NaCl, 0.8 per cent.

A drop of the suspension is deposited on a formvar film held by the support. After rinsing the film and shadowing with a mixture of Pt, Pd, Au, the electron microscope reveals the structure given by Figs. 1, 2 and 4. Many granules remain roughly spherical and are of the size expected from previous observations made *in situ* [1, 3] or by optical microscopy [13]. Some granules have lost their structure and appear as flat disks recalling, at another dimension, the ghosts of bacterial protoplasts [18, 19]. They are probably empty mitochondria. Even smaller granules (0.1–0.2 μ) are present. Whether they are mitochondria of variable size according to the cytological observations of Ephrussi [7] or that they represent submitochondrial units similar to those described by Green and his collaborators [9] in preparations of mitochondria from animal tissues is a question under study.

Individuality of the mitochondria is also given by ultrathin sections of the sediment (*M*). These particles are treated through the classical stage of dehydratation by ethanol and embedded in a mixture of methyl and butyl metacrylate (1:9) containing 0.02 per cent of benzoyl peroxide. All stages are done with careful resuspension. The polymerisation is obtained at 55°C followed by ultraviolet illumination. The sections have been obtained with a Sjöstrand ultra-microtome working with a metallic knife.


1μ
1
1μ
2
1μ
3
1μ
4

The section of the mitochondria is visible on Fig. 3. The external membrane is well defined. That the internal fine structure is not visible is probably due to the same difficulties as those encountered in similar preparations of cell-free animal mitochondria [14].

The homogeneity of the preparations and the biochemical activities of purified cytoplasmic granules are under study.

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Fig. 1.—Mitochondria isolated from yeast cells (*M*).

Fig. 2.—Mitochondria isolated from yeast cells (*M*).

Fig. 3.—Ultrathin section of isolated mitochondria (*M*).

Fig. 4.—Small granules (25–30 mμ) isolated from yeast cells (*m*).

OBSERVATIONS ON THE INCORPORATION OF ^{32}P BY IRRADIATION-PRODUCED GIANT CELLS

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Received June 26, 1958

MAMMALIAN cells in tissue culture, when irradiated with X-ray, may lose their ability to multiply while continuing to increase in size [3]. Such irradiation-produced "giants" were also reported [3] to be more susceptible than normal cells to the cytopathogenic action of Newcastle disease virus (NDV).

During an investigation of the effect of X-irradiation on virus sensitivity and yield the question arose whether these "giant" cells result from growth (i.e., the synthesis of large molecules) or from swelling due to intake of large quantities of water. One approach to an answer involves the distribution of radioactive phosphorus (^{32}P) in trichloro-acetic-acid (TCA)-soluble and -insoluble fractions of both X-irradiated and non-irradiated cells.

Trypsinized suspensions of S_3 HeLa cells[1] were prepared according to the method of Marcus *et al.* [2], suspended in growth medium and distributed in French bottles. Some of the bottles were irradiated with 1500 r, using a Machlett Industrial Thermax X-ray tube operating at 140 PKV and 9 m.a. with filtration consisting of 1/4 mm copper and 1 mm aluminum. The irradiated bottles and an equal number of non-irradiated bottles were then incubated at 37°C in an atmosphere of 5 per cent CO_2 in air.

After 3 to 4 days of incubation, the cell layers were washed with Saline A of Marcus *et al.* [2] and overlaid with a medium consisting of 10 per cent placental serum in a phosphate-free balanced salt solution. To this medium ^{32}P was added to the desired concentration. All bottles were then returned to the incubator for 48 additional hours, when the radioactive medium was removed and monolayers were washed with the phosphate-free salt solution to remove residual ^{32}P and trypsinized to remove the cells.

An aliquot was used for a cell-count and the remainder was centrifuged at 1500 rpm in an International Refrigerated Centrifuge Model PR1. The pellet was suspended in distilled water and the suspension was frozen and thawed 3 times to disrupt the cells and then placed in an ice bath. Serum at a final concentration of 3 per cent was added as a carrier for TCA precipitation. An aliquot was assayed directly for ^{32}P content and the remainder was precipitated with TCA at a final concentration of 2.5 to 3 per cent. The precipitate was then removed by centrifugation and the ^{32}P content of the supernatant was determined.

All ^{32}P determinations were made by a direct plating technique and counting in a Packard Flo-Window Counter. Plates were so prepared as to minimize self-absorption.

[1] Cell culture of clonal HeLa line S_3 was obtained through the courtesy of Dr. T. T. Puck.

TABLE I. *Effect of X-irradiation on distribution of ^{32}P between TCA-soluble and TCA-insoluble fractions of HeLa cells in tissue culture.*

Bottle	Treatment	Cell count per bottle	Amount of ^{32}P activity (counts/minute)			TCA-insoluble ^{32}P (per cent)
			Added	Incorporated into cells	TCA-soluble	
A1	1500 r	3.6×10^6	9.7×10^6	6.5×10^5	4.3×10^5	33
A2	1500 r	2.9×10^6	9.7×10^6	6.3×10^5	4.4×10^5	31
B1	No irrad.	6.3×10^6	9.7×10^6	8.7×10^5	6.0×10^5	31
B2	No irrad.	4.6×10^6	9.7×10^6	9.6×10^5	6.3×10^5	34

Results of a typical experiment are shown in Table I. In other similar experiments the TCA-insoluble fraction varied in irradiated cells between 25 and 37 per cent and in non-irradiated cells between 25 and 35 per cent.

The results seem to support the concept that an increase in the size of irradiated cells is caused by continued synthesis of large molecules (i.e., growth) in cells that have lost their ability to divide. This observation correlates with the ability of such cells to support the synthesis of poliomyelitis virus [1].

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ANTEPHASE AND DNA DOUBLING

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Received July 2, 1958

ANTEPHASE is a physiological period preceding prophase during which energy is produced and stored for the process of cell division [1]. Since DNA doubling is a prerequisite for cell division and also occurs prior to prophase [5], the question arises whether antephase is related to or includes the period of DNA doubling.

In the work reported here, the antephase period was established in mouse ear epidermis cultured *in vitro* using the same conditions from which the concept of antephase was originally formulated [3]. Microspectrophotometric absorption methods were used to determine DNA contents in individual epidermal nuclei.

Conclusions concerning DNA doubling were based upon the proposition that a diploid tissue with dividing cells contains two classes of nuclei, one of which has twice the amount of DNA in preparation for division [5]. Thus the number of epidermal nuclei in the second class could be used as a measure of DNA doubling in relation to antephase, just as the number of epidermal mitoses is used as a measure of antephase action on cell division. If antephase energy is indeed used for DNA synthesis, as well as for mitosis, there should be some correlation between DNA doubling and mitotic activity during the experimental period. It is the purpose of this study to see whether such a correlation exists.

Materials and methods.—Mouse ear epidermis was cultured *in vitro* according to the method of Bullough [2]. Adult male mice were used in groups of five, and small pieces of their ears were incubated in a Warburg flask in a saline culture medium in an oxygen gas phase at 38°C. When glucose was used it was added to the culture medium in a final concentration of 0.02 *M*.

The ear fragments were incubated for 1 hour to allow all mitoses originally present in the epidermis to pass beyond the metaphase stage. The 4 hour experimental period began after this first hour of incubation. When mitotic activity was studied, 0.016 mg colchicine was tipped into the main vessel of the Warburg flask, and the number of epidermal mitoses arrested in metaphase was determined at hourly intervals during the 4 hour experimental period.

DNA doubling was also studied at hourly intervals and during the same 4 hour experimental period. Although preliminary experiments had shown that colchicine has no effect on DNA synthesis, colchicine was not present in those experiments concerned with DNA doubling. DNA contents were determined by microspectrophotometric absorption measurements of Feulgen stained material according to the method described by Pasteels and Lison [4]. The DNA content of individual nuclei was estimated in arbitrary units by multiplying the extinction value by the nuclear area.

In each experimental group, DNA measurements were made in 100 randomly selected interphase nuclei located in the Malpighian layer of the epidermis. Frequency histograms were constructed of the amounts of DNA in individual liver and epidermal cell nuclei, and it was evident that the nuclei of the Malpighian layer fall into two distinct classes, the second of which has twice the diploid amount of DNA. Since interest centers around DNA doubling in Table I, only the number of nuclei in the second class, designated as 4C, are shown.

Results and Conclusions. Table I shows the relationship of antephase energy to both DNA doubling and to mitosis in mouse ear epidermis cultured *in vitro*. Antephase energy has been provided in the usual way [3] by culturing the ear fragments in the presence of glucose and oxygen. The presence of glucose results in a progressive and marked increase in the number of epidermal cells that enter mitosis during each hour of the 4 hour experimental period. It has been concluded by Bullough that the energy derived from glucose during antephase thus enables a greater number of epidermal cells to divide [1].

TABLE I. *The relationship of antephase energy to DNA doubling and to mitosis in mouse ear epidermis cultured at 38°C in an oxygen gas phase.*

Exptl. period (hrs.)	Saline alone		Saline + glucose	
	DNA doubling no. 4C nuclei[a]	Mitotic activity av. no. mitoses[b]	DNA doubling no. 4C nuclei[a]	Mitotic activity av. no. mitoses[c]
1	13	0.4 ± 0.03	14	2.7 ± 0.3
2	12	1.1 ± 0.3	18	4.1 ± 0.6
3	14	0.7 ± 0.09	16	7.3 ± 0.7
4	18	1.8 ± 0.7	15	8.0 ± 0.9

[a] Number of 4C nuclei (double DNA content) per 100 measurements.
[b] Average number of mitoses ± (S.E.) per 1 cm unit length. 1210 ± 38 (S.E.) nuclei per cm.
[c] Average number of mitoses ± (S.E.) per 1 cm unit length. 1287 ± 23 (S.E.) nuclei per cm.

In contrast to mitotic activity, DNA doubling is unaffected by antephase energy. The number of 4C nuclei remained approximately the same both in the presence and absence of glucose. The small number of interphase nuclei that leave the 4C condition to enter mitosis (approximately 1 per 150 cells) would not influence the relative proportions of 2C and 4C nuclei measured. Since the number of 4C nuclei remained unchanged, it was apparent that DNA doubling had not occurred at any time during the experimental period. Thus it could be concluded that antephase energy is not used for DNA doubling.

Under the present experimental conditions, DNA doubling has already taken place *in vivo* before experimental alterations in energy supply during antephase are studied *in vitro*. Thus the antephase period as conceived of from experiments with mouse ear epidermis *in vitro* is unrelated to and does not include the period of DNA synthesis.

Acknowledgement.—We wish to thank Prof. W. S. Bullough, Birkbeck College, University of London, in whose laboratory the experiments were carried out, Prof. J. Pasteels, Université Libre de Bruxelles, for the use of his microspectrophotometer, and Dr. H. S. DiStefano, State University of New York at Syracuse, on whose microspectrophotometer the DNA measurements reported here were repeated and confirmed.

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DEMONSTRATION OF BI-PARTITE SPIRAL STRUCTURE ON SPERMATOGONIAL ANAPHASE CHROMOSOMES OF *MUS MUSCULUS*[1]

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Received July 12, 1958

Spiralization which is now accepted as a universal feature of chromosome structure was first observed in 1880 by Baranetsky in pollen-mother cells of *Tradescantia* [1]. Apparently, the details of spiral structure can be visualized without much difficulty on chromosomes of such favorable materials as *Tradescantia* [6], *Trillium* [5, 8] and certain flagellate protozoa [2]. Demonstration of spiral structure of the large chromosomes of some orthopterous insects, on the other hand, appears to require special methods of fixation or pretreatment [3, 4], and to the best of our knowledge, distinct spiral structure has never been clearly visualized on chromosomes of mammals, although some spermatogonial metaphase chromosomes of *Rattus norvegicus* photographed by Tjio and Levan are highly suggestive of a spiral structure [9]. Recently, we have been able to demonstrate clear spiral structure of spermatogonial anaphase chromosomes of *Mus musculus* by the over-night refrigeration of fixed materials. Distilled water pretreated, acetic acid fixed squash preparations of seminiferous tubules prepared by the manner previously described [7] were immersed in 5 per cent acetic acid solution and kept in the refrigerator for an over-night period before they were subjected to Feulgen stain. The temperature was about 3°C.

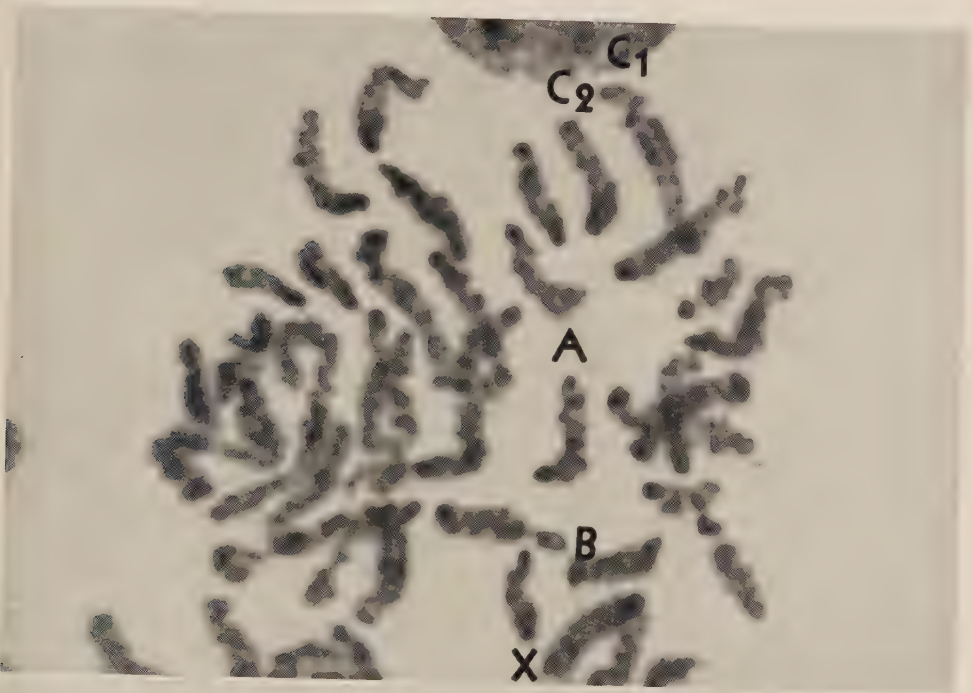
The diploid chromosome complement of *Mus musculus* consists of 40 acrocentric chromosomes, 19 homologous pairs of autosomes having a prominent block of heterochromatin adjacent to the kinetochore, while the X and Y chromosomes are almost totally heterochromatic [7]. In tissue prepared in the above manner, spermatogonial autosomes in anaphase demonstrate not only prominent centromeric heterochromatin at their proximal ends but also smaller telomeric heterochromatin at the distal ends.

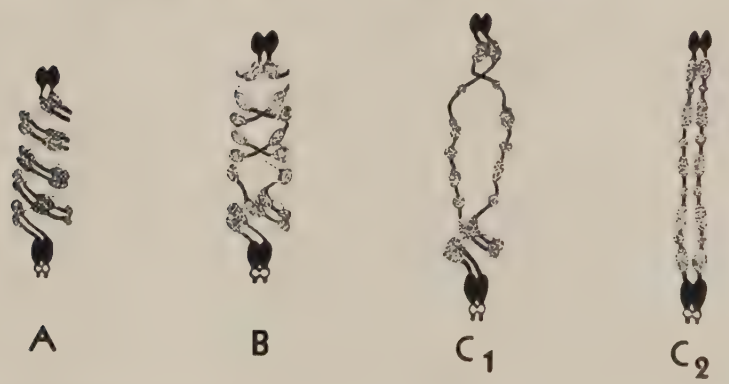
[1] Supported in part by both PHS Grant 2597 and Damon Runyon Grant 428.

Fig. 1. A photomicrograph of anaphase chromosomes (daughter chromatids) at a single division pole of the spermatogonium. Leitz Panphoto 15 × 90.

Chromosome A: A small autosome in particularly good focus. The spiral seen in the euchromatic portion between two terminal heterochromatin blocks consists of four gyres. Three minute Feulgen positive bodies arranged linearly are seen on the third gyre from the proximal end. *Chromosome B:* A second small autosome in particularly good focus. Two chromonemata in the euchromatic portion are in two independent spirals criss-crossing each other. *Chromosome* C_1 *and* C_2: Two medium-sized autosomes in good focus. Two chromonemata in the euchromatic portion are almost completely (C_1) stretched. Chromomeres may be recognized on each chromonema. *Chromosome X:* The totally and completely (C_2) heterochromatic X chromosome. The Y chromosome is not in good focus.

Schematic drawings of chromosomes A, B, C_1, C_2 are presented below the photograph.


C1
C2
A
B
X


A
B
C1
C2

Spiral structure is observed on the euchromatic portion between those two terminal heterochromatin blocks. The spiral of the largest autosome consists of about 14 gyres, while that of the smallest autosome is made of 3 gyres. Minute Feulgen positive bodies arranged linearly may occasionally be observed on the thread which forms the spiral (three such bodies may be recognized on the third gyre from the proximal end of the chromosome A of Fig. 1). From the photograph, they may appear as gyres of a minor spiral, close examination under the microscope, however, suggests them to be chromomeres on chromonemata. As was the case in *Tradescantia* [6], *Trillium* [5] and certain Orthopterous insects [10], the direction of coiling (right- or left-handed) for any particular autosome of *Mus musculus* is not constant. The highly condensed nature of the totally heterochromatic X and Y chromosomes appears to prevent spiralization of chromonemata, thus, spiral structure is not observed on either the X or the Y of spermatogonial anaphase. Shortly after daughter chromatids have reached the opposite poles at the end of anaphase, separation of the two chromonemata appears to take place. Accordingly, chromosome B of Fig. 1 demonstrates two spirals criss-crossing each other along their euchromatic portions. The bi-partite nature of anaphase chromosomes is clearly demonstrated by chromosome B of Fig. 1.

The fact that over-night refrigeration following fixation which led to the visualization of spiral structure on spermatogonial anaphase chromosomes of *Mus musculus* did not seem to diminish the over-all affinity of those chromosomes to Feulgen stain appears to indicate that the revelation of spiral structure is due to loosening of the existing spiral rather than removal of any Feulgen positive material. If this is the case, complete stretching of the chromonemata following treatment may be expected in the case of some of the anaphase chromosomes prepared in this manner. Indeed, the two chromonemata in the euchromatic portion of about 10 autosomes of Fig. 1 are completely stretched (chromosome C_1 and C_2 of Fig. 1). These artificially stretched anaphase chromosomes confirm beyond any doubt the bi-partite nature of daughter chromatids and chromomeric nature of the Feulgen positive bodies occasionally seen on the gyre of the spiral.

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PROLIFERATION OF CHICK EMBRYONIC HEART FIBROBLAST IN VARIOUS MEDIA

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Received July 15, 1958

Gothoskar, Ratnam and Ramakrishnan [3] have reported that when chick embryonic heart fibroblast tissues are grown in various media with replacement of the medium at regular intervals, the increase in proliferation is in all media accompanied by increases in the sugar uptake and micro Kjeldahl nitrogen of the proliferating speck. This observation suggested that these biochemical determinants, viz. the sugar uptake and the protein content, might enable us to differentiate proliferating, surviving and deteriorating tissues. In order to investigate this possibility, chick embryonic heart fibroblasts were grown in various media and the protein synthesis and sugar uptake were determined at different stages. The results are summarised in this communication.

A description has already been given [2] of the methods of (*a*) preparation of the cultures from the heart fibroblast tissue of 11-day old chick embryo, (*b*) cultivation of the tissue specks directly in pyrex test tube without plasma clot, and (*c*) measurement of sugar uptake and protein of the proliferating tissue.

Chick embryonic fibroblasts were grown in different media without replacement of the medium during the experimental period and the changes in the protein content and sugar uptake were determined. It was found that during proliferation there were increases in the protein content accompanied by increases in the sugar uptake. Further, there was, during survival, no change in the protein content, while increases in the sugar uptake became progressively less until, finally, during deterioration, sugar was thrown out into the medium and there was a decrease in protein content. (For convenience the amount of sugar thrown out into the medium will be referred to as sugar output, as against sugar uptake, which denotes the amount of sugar utilised from the medium.) Since the increase in sugar uptake or output, as the case may be, appears to be closely related to an increase or decrease in tissue protein, the ratio of the change in the protein content of the tissue to the change in sugar content of the medium was determined in all cases. Table I gives the values of this ratio at different stages of tissue growth in different media, the term "growth" being used in this context to cover proliferation, survival and deterioration. The values reported are based on cumulative averages of data obtained from experiments which extended over a period of six months.

It is seen from Table I that the function defined by this ratio has high values during proliferation, diminishes to zero during survival and again assumes small values during deterioration. It is further observed that the value of the function remains stationary during the peak proliferation, survival and deterioration periods for all three media. This suggests that it might be possible to standardise the values of this function for different stages of growth and in different media. Such a stand-

TABLE I. *Proliferation of chick embryonic heart fibroblast tissue in vitro in various media.*

Incubation period (days)	Ratio of change in protein content of the tissue to change in sugar content of the medium			Visual observation on the growing tissue
	Eagle's medium [1]	*M* 150 medium [4]	*M* 150—glutamine medium	
6	0.00309	0.003020	0.00359	Peak proliferation
7	0.00309	0.003020	0.00359	
8	0.00309	0.003024	0.00359	
9	0.00309	0.003024	0.00359	
10	0.00240	0.0000	0.002635	
11	0.00213	0.0000	0.0000	
12	0.00000	0.0000	0.0000	
13	0.00000	0.0000	0.0000	Survival
14	0.00180	0.0000	0.0000	
15	0.00150	0.0000	0.0000	
16	0.00150	0.0000	0.0000	
17	0.00140	0.00104	0.00293	
18	0.00138	0.00102	0.00291	
19	0.00137	0.00100	0.00160	
20	0.00135	0.00101	0.00160	Steady deterioration
21	0.00135	0.00101	0.00160	
22	0.00135	0.00100	0.00160	
23	0.00135	0.00101	0.00160	
24	0.00135	0.00101	0.00160	

ardisation, if achieved, would be extremely valuable from the point of view of a quantitative differentiation of different tissues. Further work is being directed towards this end.

The data yielding these results will be more fully reported in the *Indian Journal of Medical Research.*

Acknowledgement. –The authors wish to express their thanks to the Indian Council of Medical Research for sponsoring this research project and La Roche & Co., England, for the valuable gift of vitamins used in these investigations.

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ACTION MORPHOGÉNÉTIQUE DE L'URÉE SUR LE TRYPANOSOME

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Reçu le 20 juillet, 1958

L'étude du déterminisme de la cytodifférenciation chez les métazoaires se heurte à des difficultés inhérentes à la complexité du matériel étudié : les interactions réciproques des cellules qui constituent un tissu ou un territoire présomptif, de même que l'hétérogénéité de ces derniers, sont parmi les principales causes d'insuccès.

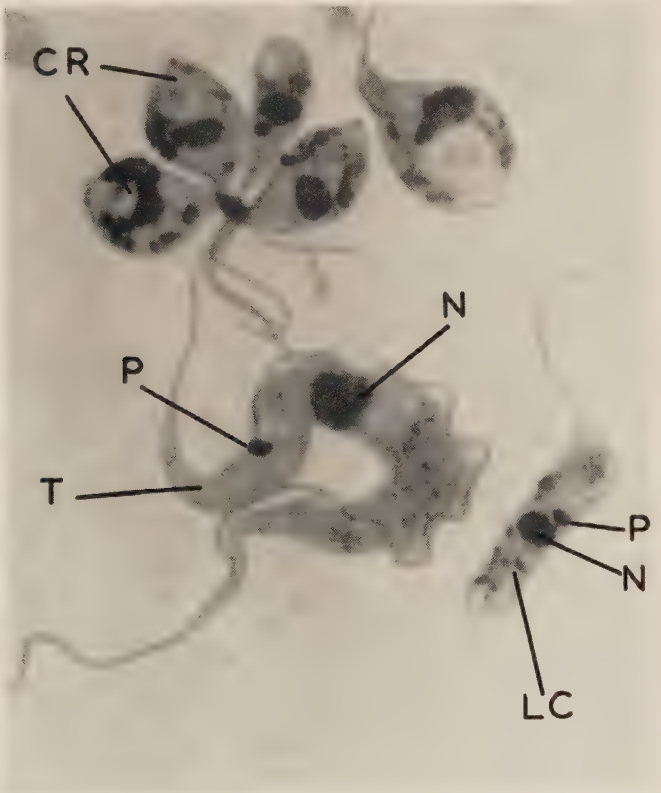

Fig. 1.—*Trypanosoma mega* cultivé pendant trois jours dans le milieu + urée (1 mg/ml). *CR*, Crithidias en rosace issues d'un individu non sensible; *LC*, Crithidia libre; *T*, Trypanosome de type sanguicole, transformé à partir d'une crithidia sensible; *P*, Corps parabasal; et *N*, Noyau. Frottis coloré au Giemsa.

D'autre part l'extrême dépendance des cellules d'un être organisé rend leur culture délicate et la reproduction *in vitro* des conditions naturelles de milieu et d'environnement est pratiquement impossible. Il nous a paru séduisant d'aborder le problème chez un protozoaire, comme le trypanosome, dont les transformations morphologiques sont bien connues.

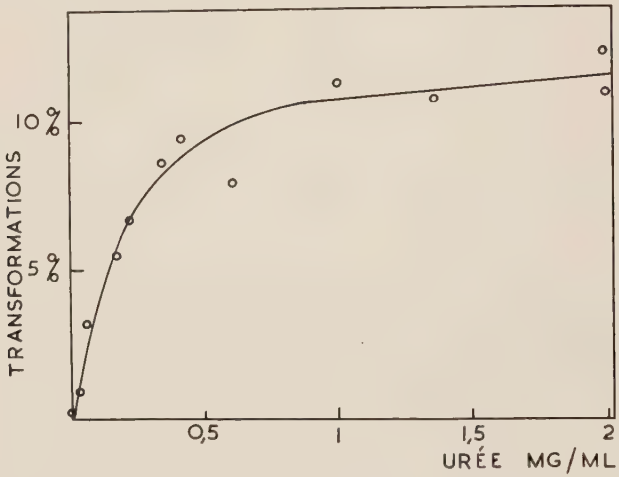

Fig. 2.—Augmentation du pourcentage de transformations pour une même culture compétente inoculée dans des milieux de teneur croissante en urée.

Nous avions montré, dans des publications antérieures [7, 5]. que le passage de la forme crithidienne à la forme sanguicole de *Trypanosoma mega* est régie par au moins deux facteurs : l'un a été décrit comme une substance morphogénétique présente dans le sérum des vertébrés; l'autre, endogène au flagellé, détermine la sensibilité de ce dernier à la substance morphogénétique du sérum. Nous avions aussi défini certaines propriétés essentielles du facteur sérique [5], comme sa résistance à l'ébullition et à la trypsine qui faisaient prévoir sa nature non-protéinique.

Des essais systématiques d'isolement de ce facteur morphogénétique à partir de sérum de bœuf déprotéinisé par l'ébullition nous ont finalement donné un produit pur, recristallisé plusieurs fois dans l'acétone et dont l'activité transformante spécifique (rapportée au poids sec) est environ cent fois plus élevée que celle du sérum. L'identification de cette substance fut facile : il s'agissait d'urée.

Nous avons incubé du sérum déprotéinisé avec de l'uréase (Sigma Chemical Co., type II—1 mg/ml) pendant 90 minutes à 37°C. Ce traitement détruit le facteur transformant. Le rôle de l'urée dans la transformation du trypanosome, fort inattendu, fut encore confirmé en provoquant des transformations avec de l'urée du commerce. Des crithidias d'une culture compétente ont été inoculées dans du milieu bacto-tryptose [5] additionné d'urée (Fisher certified reagent) à diverses concentrations. Le résultat de cette expérience est illustré par les figures 1 et 2. Le pourcentage de transformations augmente avec la concentration de l'urée et atteint un palier à

partir de 0,40 mg d'urée par ml de milieu. La courbe est très semblable à celle que l'on obtient avec du sérum [5] au lieu d'urée.

Nous avions observé antérieurement que le pouvoir transformant du sérum paraissait lié aux globulines [7] et aussi qu'une préparation commerciale de sérum-albumine (Armour, fract. V) pouvait provoquer la transformation de *T. mega* [6]. Bien que ces observations soient en apparente contradiction avec celles que nous présentons ici, elles n'impliquent pas nécessairement l'existence dans le sérum d'un second facteur transformant, différent de l'urée. On sait en effet que de grandes quantités d'urée peuvent être adsorbées par les sérumglobulines [4] et les sérum-albumines [1, 2, 4]. D'autre part nous avons pu complètement inactiver le sérum par une dialyse prolongée pendant trois ou quatre jours contre de l'eau courante.

Aucune hypothèse relative au mécanisme d'action de l'urée sur le trypanosome ne peut être actuellement avancée. Il semble cependant que l'étude de ce mécanisme d'action puisse recevoir une double orientation. Il faudrait étudier d'une part une intervention éventuelle de l'urée dans le métabolisme azoté du flagellé, soit comme métabolite, soit comme inhibiteur. On pourrait envisager d'autre part une action physique de l'urée sur les protéines. On sait par exemple que la myosine subit des modifications de structure sous l'action de concentrations relativement faibles d'urée [3]. Remarquons cependant que d'une manière générale la dénaturation des protéines s'obtient avec une très forte concentration en urée, de l'ordre de 4 à 8 *M*, soit plusieurs centaines de fois plus élevée que celle qui induit la transformation du trypanosome.

Des expériences sont en cours dans ce laboratoire qui permettront peut-être de choisir entre ces deux orientations possibles.

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AUTORADIOGRAPHIC EVIDENCE OF DISTRIBUTION OF ^{35}S-METHIONINE IN EHRLICH ASCITES CELLS

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Received July 28, 1958

The purpose of the present paper is to report some histoautoradiographic observations on the cells of the Ehrlich ascites tumour which add further information to our previous biochemical investigations [2].

The source of our material was 6 days old tumours from twelve albino mice. The animals were injected intravenously with 10 μc of ^{35}S-DL-methionine (containing 1 mc/mM). The animals were killed 15, 30, 60 and 180 minutes after the inoculation of the isotope and the ascitic fluids were collected immediately. Four smears on glass slides were prepared from each specimen and fixed in absolute methyl alcohol for 2 minutes. Then the slides were coated in the usual way with the stripping film (Kodak AR 10). The films were exposed for 15 days and, after development, stained with carmalum. A number of controls were prepared with ascitic fluid of mice which had not been injected with the radioactive compound. No traces appeared in them after 15 days of exposure.

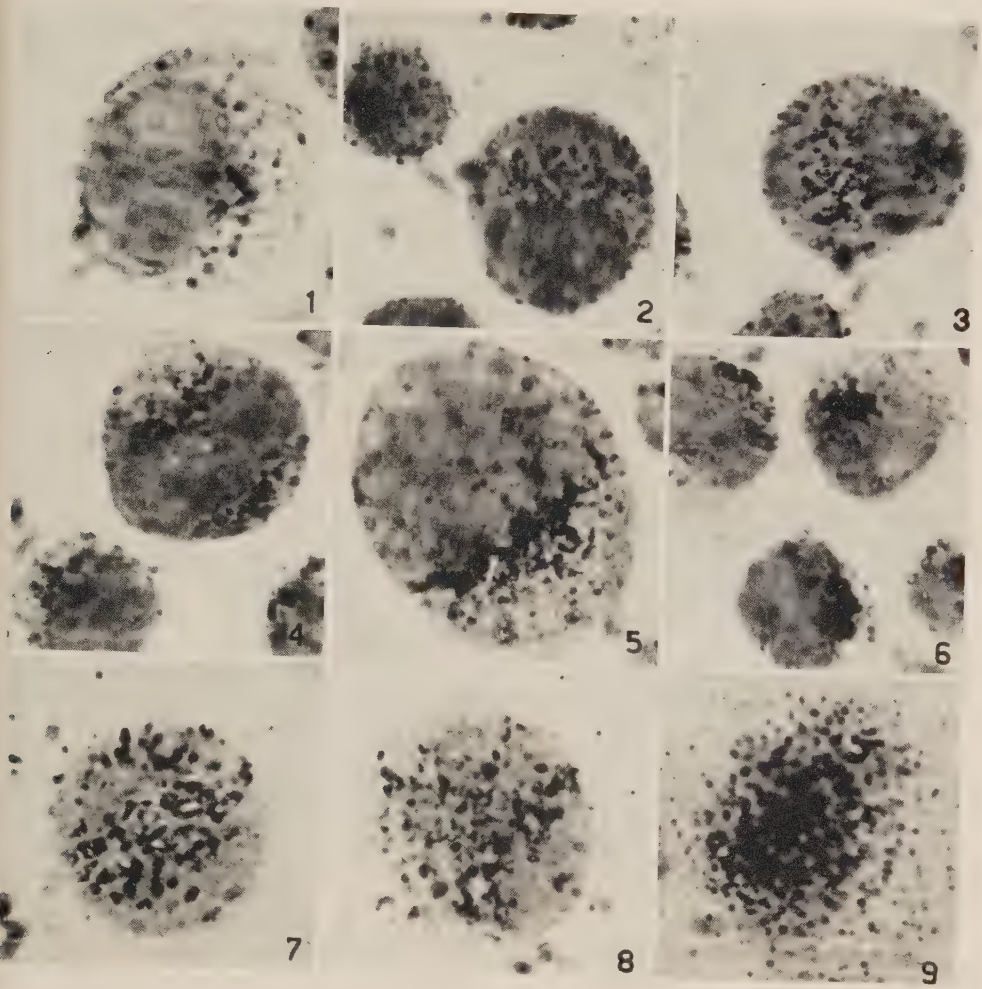
In all our autoradiographic experiments only very few traces were found 15 minutes after the injection of ^{35}S-DL-methionine (Fig. 1). After 30 minutes, the incorporation was quite good; traces were found almost exclusively in the cytoplasm (Figs. 2 6). Only a slight radioactivity appeared in the nuclei and in most cells the greatest density was close to one pole of the nucleus. A similar observation has been made by Sirlin and Waddington [2] on the cells of the chick embryo to which ^{35}S-methionine had been administered. This territory is known to be rich in ribonucleoproteins and is usually considered to be a site of active protein synthesis.

Smears of ascites cells collected 180 minutes after the administration of the isotope show scattered cells exhibiting a very heavy labeling of the nucleus (Figs. 7–8). In most cells, however, the number of traces in the nucleus is very small. In the former cells the heavy activity in the nucleus entirely masks the cytoplasmic activity (Fig. 9), whereas in the latter the situation is similar to that found in the 30 minute experiments.

On *in vitro* experiments it was shown by Allfrey *et al.* [1] that, in contrast to thymus nuclei, isolated nuclei of lymphoma cells incorporate very little ^{14}C-alanine in their proteins. Preliminary experiments in which we have used ^{35}S-methionine and isolated nuclei of ascites cells have confirmed this observation.

The present observations may suggest that such an apparently low level of incorporation of labeled amino acids in the nuclear proteins may depend on the fact that only a small proportion of nuclei of tumour cells are able to accomplish such incorporation, thus resulting in a strong dilution of the activity.

This non-uniform behaviour of the nuclei of the Ehrlich ascites cells as indicated



Figs. 1–9.—Ehrlich ascites cells after intravenous injection of 10 μc of ^{35}S-DL-methionine. *Fig. 1*, after 15 minutes; *Figs. 2–6*, after 30 minutes; *Figs. 7–9*, after 180 minutes.

by their different ability to incorporate labeled amino acids is of interest as it may be a physiological counterpart of the morphological heterogenicity of these cells and in particular of their nucleus.

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A SIMPLE MONOCHROMATOR FOR ULTRAVIOLET MICROSCOPY

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Received August 10, 1958

In most commercial monochromators the aperture of the exit beam is much larger than that of a microscope condenser in its object space. Accordingly, unless extra mirrors or lenses are used, a considerable amount of light is lost and the dispersive power of the instrument is not fully utilized (e.g. [1, 2]).

We have found that a very useful and extremely simple monochromator can be built with an aperture ratio of the exit beam of 1:70 if the parts are arranged as in Fig. 1. There is one concave spherical mirror S_1 which yields an image of the entrance slit in the plane of the exit slit. Of the rays passing through the prism those nearest to the refracting rib are made to be minimally refracted. As the beam is converging, the other rays will undergo an ever greater than minimal deviation according as their distance from the refracting rib increases. It has been found that this results in a remarkably good compensation of the image faults caused by the off-axis position of the spherical mirror. For the purpose in hand the effectiveness of this compensation is amply sufficient throughout the visible and ultraviolet spectra as is evidenced by the high pressure mercury arc line images in Figs. 2 and 3. The complete reversal of the mercury line 254 mμ that occurs in the arc we used may serve to give an estimate of the amount of stray light present in the ultraviolet part of the spectrum (see Fig. 3). The relative intensities of the spectrum have been measured by means of a 1 P 28 RCA photomultiplier tube. It was found that at the 254 mμ setting of the monochromator, the photomultiplier current was less than 1 per cent of that at either 248 or 257 mμ. Apparently, the amount of stray light in the monochromator is negligible when used with a mercury arc.

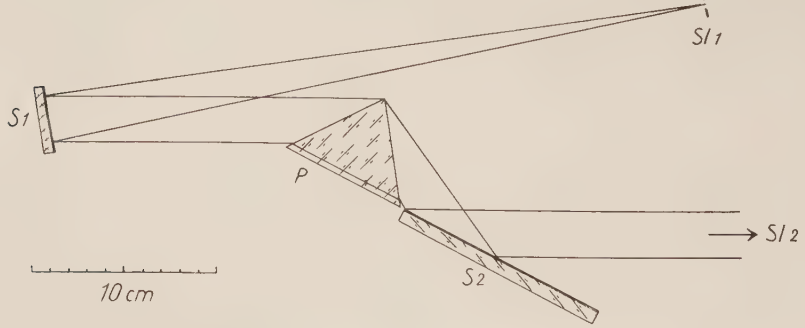

Fig. 1.— Diagram of the monochromator. The greater part of the exit beam and the exit slit have been omitted. Sl_1, entrance slit; S_1, concave spherical mirror; P, prism; S_2, flat mirror, → Sl_2, towards exit slit at 200 cm from S_1.

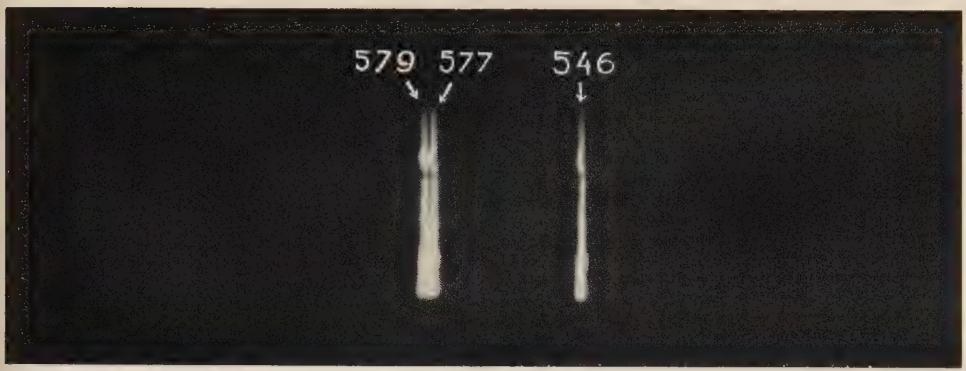

Fig. 2.—Part of the visible spectre photographed by substituting a plate for the exit slit of the monochromator. Numbers denote wavelenghts in mμ.

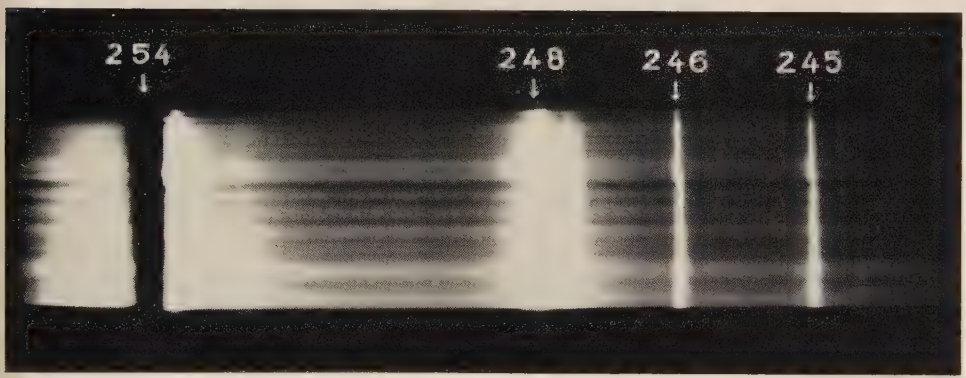

Fig. 3.—Part of the ultraviolet spectre. Though heavily overexposed the reversed line at 254 mμ is completely dark.

The skillful assistance of Mr. W. Kerssen, who built the mechanical parts of the monochromator, is gratefully acknowledged.

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CONTRACTILE ELEMENTS IN THE LIMITING MEMBRANE OF THE SEMINIFEROUS TUBULES OF THE RAT[1]

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Received August 15, 1958

The seminiferous tubule of the rat is limited by a complex 1 μ-thick membrane, which has been investigated as early as 1871 by von Ebner [1]. The description was completed by Regaud [4], who observed two concentric lamellae composed of a homogeneous substance or "substance fondamentale", enclosing a space occupied by a continuous single layer of flattened cells. This author showed that, after staining with silver nitrate, the cytoplasmic limits of these cells showed a polygonal pattern similar to that observed in endothelia. He considered these elements to be modified connective tissue cells [4].

When the limiting membrane was examined in sections stained with the periodic acid-Schiff technique, the two concentric lamellae but not the interlamellar space showed the intense staining characteristic of most basement membranes (Fig. 1). In places, the lamella which is in direct contact with the Sertoli cells and spermatogonia (or internal lamella) appeared to be split into two reactive layers.

With the electron microscope, the internal lamella showed three distinct layers (Fig. 2): an inner layer composed of homogeneous material of moderate density to the electron beam, an intermediate layer containing many collagenous fibrils running more or less parallel to the axis of the tubule, and an outer layer composed of homogeneous material similar to that of the inner layer.

The external lamella (on the side of the interstitial tissue) was found to be composed of homogeneous material and of a few collagenous fibrils. At the surface of the external lamella and closely applied to it, there were connective tissue cells with flattened nuclei and an extremely thin cytoplasmic body (containing mitochondria, rough and smooth surface vesicles) which formed an almost continuous sheath on the outside of the tubules, except for occasional small gaps between the edges of two adjacent cells. The three layers of homogeneous material—two in the internal, and one in the external lamella—are presumably the PA-Schiff reactive layers seen with the light microscope.

The space between internal and external lamellae was occupied by the cytoplasm of the interlamellar cells. At the point of contact between two of these cells, the cytoplasmic membranes were closely apposed to each other, leaving no gap. In places, the two adjacent membranes thickened and showed dense material which bears some resemblance to the intercalated discs of heart muscle fibers [2]. Half way between the two opposite cell surfaces, the cytoplasm of the interlamellar cells showed relatively few organelles (mitochondria, aggregates of dense granules, rough surfaced vesicles, and smooth surfaced Golgi type vesicles) most of which were found half way between the two cell surfaces. The cytoplasmic membranes facing the internal and external lamellae showed numerous small, smooth surfaced invaginations similar to those described in the endothelial cells of blood capillaries [3]. In

[1] This study was aided by a grant from the Population Council, New York, New York.

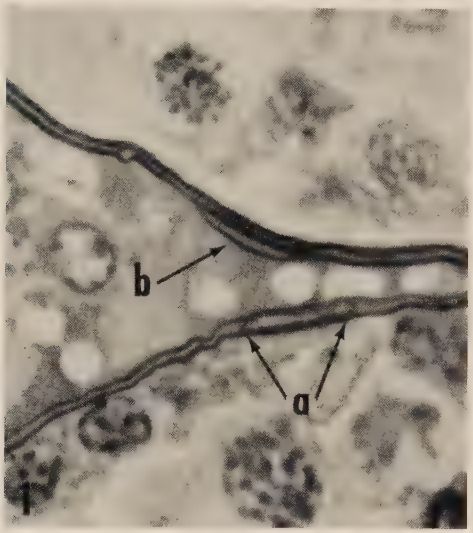

Fig. 1.—High power microphotograph (× 1600) of a section of rat testis stained with the periodic acid-Schiff hematoxylin technique. The photograph shows portions of two seminiferous tubules demarcated by their respective limiting membranes and separated by some interstitial tissue. In each limiting membrane two PA-Schiff positive lamellae may be seen: the internal lamella (seminiferous epithelium side) and the external lamella (connective tissue side). The nucleus of an interlamellar cell is seen at *a* between the tips of the arrows. The nucleus of a connective tissue cell flattened along the external lamella is indicated by arrow *b*.

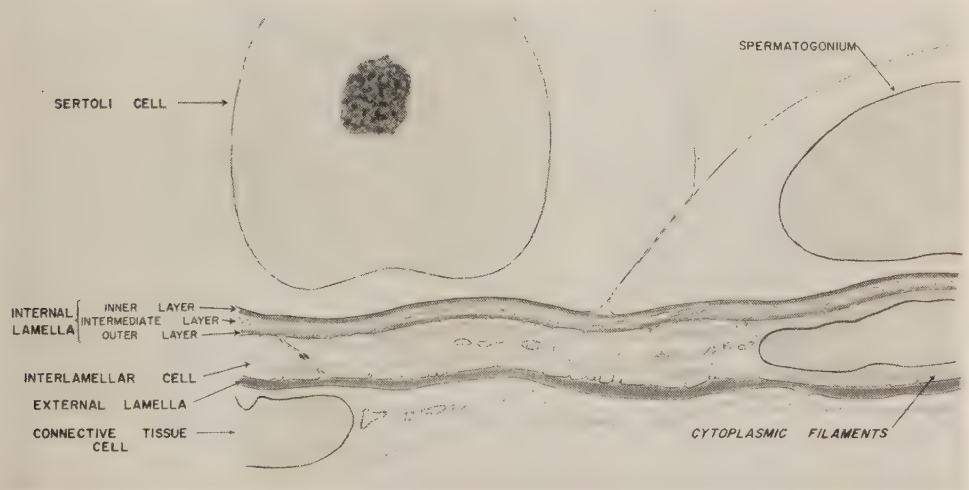

Fig. 2.—Diagram representing the limiting membrane of the seminiferous tubule of the rat, as seen with the electron microscope.

addition to these organelles, the cytoplasm of interlamellar cells contained dense meshworks of extremely fine filaments running in bundles along the cytoplasmic membrane (Fig. 2). When seen in cross and oblique section these filaments gave a hairy appearance to the cytoplasm.

The main features of interlamellar cells, that is, the central location of organelles, the invaginations at the cell surfaces, and particularly the cytoplasmic filaments, all combined to make the interlamellar cells somewhat similar to smooth muscle fibers [6]. Perhaps the homogeneous material of the external lamella and that of the outer layer of the internal lamella correspond to the homogeneous material —sarcolemma—coating smooth muscle cells. (The inner layer of the internal lamella would thus be the true basement membrane of the seminiferous epithelium.)

The presence of cells resembling smooth muscle fibers reminded us of the finding of contractions of the seminiferous tubules in the rat and dog by Roosen-Runge [5]. Examination under high magnification ($\times$ 400) of seminiferous tubules of living rats in a Ringer solution at 25–36°C confirmed the existence of contractions along the length of the tubules. These contractions had a periodicity of 30–45 seconds and caused depressions of the tubular wall which varied in amplitude up to a maximum of 10 μ in depth. These contractions travelled over short distances and seemed to move in the direction of the rete testis as suggested by the examination of the terminal portions of the tubules.

Since the contractions of the tubules had been attributed to Sertoli cells [5], an attempt was made to find out whether these contractions originated in the seminiferous epithelium or in the limiting membrane. By means of light pressure, it was found possible to displace the cells of the seminiferous epithelium from short portions of tubules, so that the limiting membrane became free of germinal and Sertoli elements. In such areas, the limiting membrane showed clearcut contractions having the same periodicity as in intact tubules. The persistence of contractions of the tubular wall in the absence of germinal and Sertoli cells demonstrated that the contractile elements are located in the wall itself. Since furthermore the interlamellar cells displayed filaments and other morphological features similar to those found in smooth muscle cells, the contractions of the tubules were attributed to the interlamellar cells.

While the location of these cells on the outer surface of the seminiferous epithelium would tend to classify them as myo-epithelial cells, their plate-like shape [4], their arrangement as a continuous mono-cellular layer and their inclusion between two basement membranes make them quite different from typical myo-epithelial (basket) cells. They may be considered as a new type of contractile epithelial cell.

Since the contractions of the interlamellar cells induce frequently recurring movement of the seminiferous epithelium, they may play a role in the release of spermatozoa from the epithelium and in their transportation to the rete testis.

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6. This resemblance was kindly pointed out to us by Dr. S. Palay.

RÖNTGENHISTORADIOGRAFISCHE UNTERSUCHUNGEN AN INSELZELLEN DES PANKREAS NACH STIMULIERUNG MIT DEM ORALEN ANTIDIABETICUM D 860

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Eingegangen am 20. August 1958

Die Röntgenhistoradiographie [3, 4] ermöglicht durch Trockengewichtsbestimmungen, Rückschlüsse auf die bei verschiedenen Funktionszuständen der Zelle aus- und eintretenden Substanzen zu ziehen. In früheren Untersuchungen [6] hatten wir gesehen, dass 1½ Std. nach Gabe von D 860 (Sulfonylharnstoff, orales Antidiabeticum) der chemisch bestimmte Insulingehalt der B Zellen des Kälberpankreas um etwa 75 % absinkt, gleichzeitig die nach Gomori färbbaren Granula verschwinden, und die Reaktion für SS und SH Gruppen [1, 2] vermindert ist. (Abnahme der mittleren Extinktion von 0,98 auf 0,46 [7].) Bisher besteht aber noch kein Anhaltspunkt über die Grössenordnung der in den Inselzellen des Pankreas vorhandenen absoluten Substanzmengen. Vorliegende Untersuchungen sollen einen Beitrag zu dieser Frage liefern.

Methodik.—8 Wochen alte Kälber wurden 1½ Std. vor dem Tod mit 250 mg D 860 pro Kilogramm Körpergewicht behandelt. Pankreasstücke von behandelten und unbehandelten Tieren wurden 6 Std. in Bouinscher Lösung fixiert, zusammen in einem Paraffinblock eingebettet und mit einem sorgfältig überprüften Mikrotom-

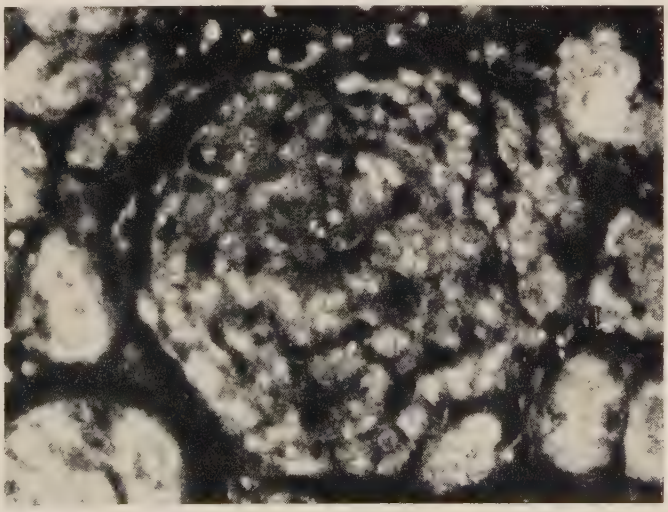

Abb. 1.—Röntgenhistoradiographische Aufnahme einer Langerhansschen Insel von Kälberpankreas bei 400facher Vergrösserung.

Tabelle I.

A = Konzentration 10^{-12} g/μ^2; B = Konzentration 10^{-12} g/μ^3 bei einer gemessenen Dicke von 5,2 $\mu \pm 0{,}1$.

	UNBEHANDELT					BEHANDELT			
	Exkretorisch Cytoplasma[a]		Inseln Cytoplasma[b]			Exkretorisch Cytoplasma[a]		Inseln Cytoplasma[b]	
	A	B	A	B		A	B	A	B
Nr. 75_1	2,65	0,51	1,68	0,325	Nr. 76_1	2,25	0,430	0,95	0,182
			1,75	0,335				1,45	0,278
			1,45	0,280				1,58	0,365
			1,33	0,257				1,70	0,327
			1,51	0,290					
			1,49	0,287	76_2	2,12	0,404	1,03	0,205
			1,52	0,293					
					77_1	2,52	0,485	1,12	0,215
								2,06	0,395
								1,33	0,255
								1,56	0,300
								2,03	0,395
								1,68	0,322
								1,38	0,265
								1,07	0,205

[a] Sämtliche gemessenen Plasmakonzentrationen wurden zu jeweils einem Mittelwert zusammen gezogen.

[b] Jeder Wert entspricht dem Durchschnitt der Konzentration aller Zellplasmen einer Insel.

messer (Scharten) geschnitten, sodass der Gewebeschnitt eines behandelten und eines unbehandelten Tieres in gleicher Dicke von 5 μ vorliegt. (Dickenbestimmung durch Fokusierung.) Die Schnitte wurden entparaffiniert, in Alkohol fixiert und auf Präparateträgern über eine Mylarfolie aufgezogen. Auf 5 Präparateträgern konnten so 13 Inseln behandelter und 7 unbehandelter Tiere untersucht werden. Röntgenkontaktaufnahmen mit dem Mikroradiographen von Philips Typ CMR 5 wurden auf „Kodak Maximum Resolution Plates" bei einer Röntgenspannung von 1,55 KV (entspricht 8 12 Å) einem Strom von 2 mA und 15 Min. Belichtungsdauer angefertigt. (Vakuum der Filmkasette besser als 0,2 Torr.) Die Platten wurden 6 Min. mit Entwickler G 209 A bei 18° entwickelt. Die belichteten Filme wurden in Eukitt eingedeckt, und die Schwärzung mit einem Cytophotometer [8] mit einer Messblende von 1,4 μ Durchmesser bestimmt. Nach einmaliger Aufstellung einer Zeitschwärzungskurve für den verwandten Film lässt sich bei der Gültigkeit des Bunsen-Roscoeschen Gesetzes auch für weiche Röntgenstrahlen von der Zeit auf die Intensität schliessen. Daraus lässt sich die Extinction des Präparates an der untersuchten Stelle gegenüber

dem verwandten weichen Röntgenlicht und damit die Trockengewichtskonzentration errechnen. (Nähere technische Einzelheiten siehe [4, 5].)

Ergebnisse.—Die Konzentration des Cytoplasmas aller Zellen einer Insel wurden zu einem Mittelwert zusammengefasst, da sich auf Grund des Röntgenabsorptionsbildes keine Differenzierung der Zelltypen ermöglichen lässt. Das Cytoplasma der Langerhansschen Inseln zeigt 60 % der Trockengewichtskonzentration des exkretorischen Gewebes (Abb. 1). Zwischen den Inseln behandelter und unbehandelter Tiere finden sich keine sicheren Trockengewichtsunterschiede (Tab. I). Nach unseren Untersuchungen [6] sinkt der durchschnittliche Insulingehalt des Gesamtpankreasextraktes 1½ Std. nach D 860 Behandlung auf 25 % ab. Da Konzentrationsunterschiede von etwa 10 % noch messbar wären, muss man schliessen, dass die Menge des ausgeschütteten Insulins weniger als 10 % des Cytoplasmagewichtes der Zellen beträgt. Es fiel auf, dass das Cytoplasma der Inselzellen unbehandelter Tiere gleichmässiger in ihrer Schwärzung erschien, als das behandelter Tiere. Mit Vorbehalten könnte man daraus ein verschiedenartiges Verhalten der einzelnen Zelltypen einer Insel vermuten.

Zusammenfassung.—Röntgenhistoradiographische Untersuchungen an Langerhansschen Inseln von Kälberpankreas ergaben, dass das Trockengewicht des Cytoplasmas der Inselzellen etwa 40 % niedriger ist, als das des exkretorischen Parenchyms. Eine Stimulierung der Inseln mit einem Insulinverlust der B Zellen ergibt keinen messbaren Trockengewichtsunterschied.

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IDENTIFICATION OF THE NUCLEUS OF A FISSION YEAST WITH FLUORESCENT DYES

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Received August 20, 1958

The fission yeast *Schizosaccharomyces pombe* has recently become an important cell for studying cell growth, because the diameter is essentially uniform and the increase in volume can be determined by length measurements. The patterns of increase in dry mass [6] and ribonucleic acid content [7] during the cell-division cycle have been measured, and various other studies are now in progress in this laboratory. This yeast contains a single central vacuole of low refractive index, which may be seen with phase contrast microscopy when the cell is mounted in media of high refractive index. Since the vacuole divides just prior to cytoplasmic division, Mitchison [6] postulated that it might be the nucleus. Two reports of studies with the Feulgen procedure and a variant on the technique [3, 5] indicated that the body contained DNA; however, workers in this laboratory (cf. [6]) found the results of the standard Feulgen procedure quite variable on this material. It was necessary, therefore, to establish conclusively whether or not the central vacuole contained DNA, and was, in fact, the nucleus. Furthermore, various studies on the cell require a simple method for identifying the nucleus. Hence, a number of standard methods and variations were attempted including fluorescent dyes which have been used successfully on mammalian tissues [1, 2].

Cells from logarithmic or resting phase cultures of *Schizosaccharomyces pombe* Lindner (N.C.Y.C. 132) grown in 2 per cent Oxoid Wort Broth were partially air-dried on slides, fixed in glacial acetic acid-ethanol (1:3), washed in ethanol, and air-dried. The slides were placed in pH 4.5 acetic acid -sodium acetate buffer for fifteen minutes and were then stained in the buffer solutions containing between 10^{-3} and 10^{-5} g/ml of either acridine orange (Hopkin and Williams, Ltd.) or trypaflavin (Bayer). The slides were rinsed in buffer and vasoline-ringed wet mounts were prepared. Since the intensity was unexplainably variable a series of staining times ranging from thirty minutes to two hours, and rinsing times from five minutes to twenty-five minutes were employed regularly.

Distilled water was often an adequate medium for all steps, but the results varied in specificity with the dye concentration. Phosphate and phthalate buffers were unsatisfactory. Living cells could be placed directly in distilled water solutions of the dye with good results, if there were not too many storage granules present.

The slides were illuminated with a high pressure mercury arc lamp (Mazda 2502), a Corning 5113 filter being used which transmits the blue to near ultra-violet spectrum.

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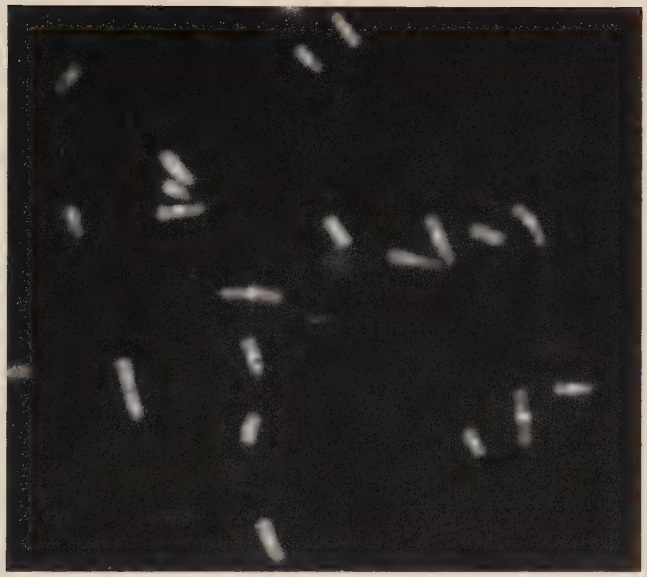

Fig. 1.—*Schizosaccharomyces pombe.* Fluorescent image of cells stained with acridine orange. Photographed through an imperfect green filter which transmits the green nuclear stain, the natural green fluorescence of the cells, and a small fraction of the orange cytoplasmic stain. The diameter of the cells equals approximately 3 microns.

A yellow filter (Cooke, Troughton & Simms M1688) was mounted in the eyepiece to absorb the illuminating beam.

Acridine-orange staining produced a brilliant orange fluorescence in the cytoplasm and an intense green, yellow-green, or yellow fluorescence in the central vacuole (Fig. 1). Numerous studies have shown that these colors are specific for RNA and DNA respectively in animal tissues [1, 2]. The specificity in this material was verified by treatment with both desoxyribonuclease and ribonuclease used according to the standard procedures [8]. Hence, the central vacuole contains DNA. Less striking results were obtained with trypaflavin, but in view of the excellent staining with acridine orange it seemed superfluous to attempt to establish the optimum conditions for trypaflavin.

Both living and fixed cells have a faint natural green fluorescence, which explains the cytoplasmic background color when the stained cell is viewed through a green filter (Fig. 1). The natural fluorescence was generally paler in the nucleus, but sometimes the unstained cell appeared uniform.

This staining technique was also tested on a few other cells that are not generally amenable to the standard Feulgen procedure. The nucleus of *Saccharomyces cerevisseae* was readily demonstrated, as has been shown previously with this dye [4]. The nucleus of the small soil amoeba *Hartmanella rhysodes* stood out clearly after staining even against some natural cytoplasmic background of the rounded cell

when viewed through a green filter. Unidentified rod-shaped bacteria contaminating one series also showed green nuclear bodies and orange cytoplasm.

These tests in comparison with the Feulgen procedure, acid fuchsin, and various basic dyes, suggest that acridine orange is the most sensitive cytochemical method for demonstrating DNA. This technique must be used with considerable attention to controls, however, because several cell-types have a faint natural fluorescence with a similar spectrum to the fluorescence of stained DNA.

Since this sensitive method has shown that the central vacuole of *S. pombe* contains DNA, it may be concluded that the central vacuole is the nucleus.

Acknowledgement.—I wish to thank Dr. J. M. Mitchison for his enthusiastic encouragement and many helpful suggestions. I also wish to thank Mr. R. A. Fox for his skilful aid in preparing the photomicrographs.

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SUBCELLULAR FRACTIONS FROM THE ELECTRIC TISSUE OF *TORPEDO*—MORPHOLOGICAL ASPECT AND CHOLINESTERASE CONTENT

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Received August 20, 1958

The cytological location of cholinesterase (ChE) is of obvious interest for understanding the mechanism of its physiological activity. From this aspect the electric tissue of *Torpedo* is particularly worthy of investigation, because of its homogeneous histological structure, highly specialized for electrogenesis, and of its high content of ChE. An earlier histochemical investigation of the electric organ of *Electrophorus* [2] showed the presence of ChE on the caudal face of the electroplaques, where nerve endings and electrogenesis have also been localized. This work, however, left un-

settled the question of the presence of ChE in other parts of the electroplaques and of its location on well-defined cytological structures.

We have prepared sub-cellular fractions from the electric tissue of *Torpedo* and examined their morphology and their ChE activity. The main aim was to obtain information about the essential distribution of ChE in recognized microscopical and/or submicroscopical structures from the electroplaques, examined by light and electron microscopy. An electron microscopical investigation on electric tissue has been briefly reported by Luft [4].

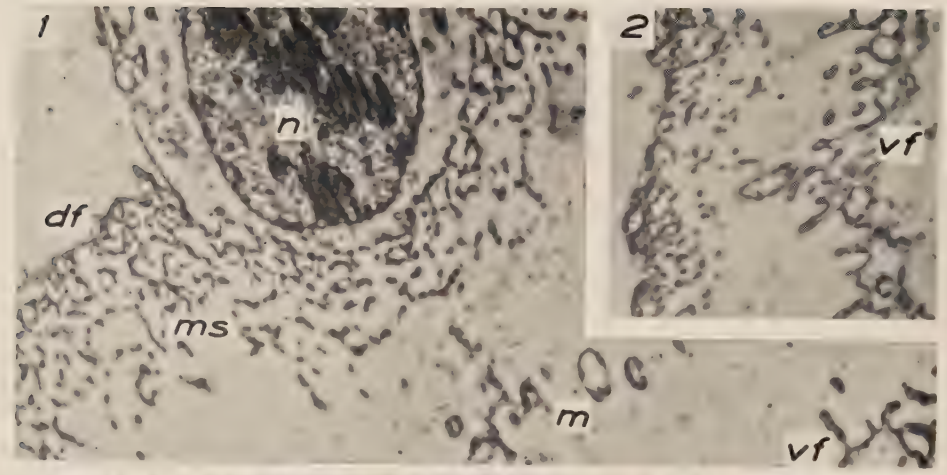
Electric tissue was excised from living specimens of *Torpedo ocellata*, then finely minced with a Latapie mincer and homogenized in a Potter-Elvehjem homogenizer with ten volumes of 0.25 (or 0.88) *M* sucrose. The homogenate was submitted to differential centrifugation and specimens from the fractions obtained were fixed with 1 per cent OsO_4 in sucrose solution and embedded in paraffin or in butyl methacrylate [5]. Sections from the paraffin-embedded specimens were stained and examined by light microscope, whereas ultrathin sections were obtained from the plastic-embedded ones by a Porter-Blum microtome and examined by electron microscope (the model worked out at Istituto Superiore di Sanità [1]).

A series of pilot experiments was made in order to find the centrifugation procedure which allows the separation of well-defined fractions which are also as homogeneous as possible. The sub-cellular fractions were compared with samples of the intact tissue which were processed and examined in the same way.

The intact tissue shows, by light microscopy, a layer of thin rodlets aligned in the cytoplasm beneath the electrolemma, mainly at the dorsal face of the electroplaques. Electron microscopical examination shows, in the same zone, the presence of thin submicroscopic membranes arranged to form a system of tubules and vesicles, which is apparently continuous (Figs. 1 and 2). This system of membranes is located close to the cell surface, mainly at the dorsal side of the electroplaque, and extends deeply in the cytoplasm. According to Luft [4] it is formed by invaginations of the plasma membrane. The opposite face of the electroplaque shows single invaginations of the cell surface. These aspects vary somewhat in the different zones of the organ. Nuclei and mitochondria are recognized both by their microscopical and submicroscopical aspect, although mitochondria appear altered by swelling. The electroplaques show a large cytoplasmic space containing some sparse granular material, not well defined at this magnification.

The examination of the sub-cellular fractions from several preparations shows that large fragments of the membrane system, forming clumps of rounded vesicles, are the main constituent of the first sedimented fraction, *A* (Fig. 3). This fraction was spun down at 800 × *g* in 15 min and contained also the nuclei and some fragments of thicker dense membranes, probably from the basal membrane of the electrolemma.

The smaller membrane fragments, forming vesicles of various sizes, were recovered in the next sedimenting fractions. Thus fraction *B*, which sedimented in the second centrifugation step, at 3000 × *g* for 15 min, is characterized by the presence of a high amount of mitochondria, recognized both by light and electron microscopy and apparently swollen, but also shows a high content of small vesicles (Fig. 4). A better separation and preservation of mitochondria was obtained in 0.88 *M* sucrose. When these mitochondrial fractions were incubated in Warburg apparatus in a suitable medium consistent rates of respiration were observed.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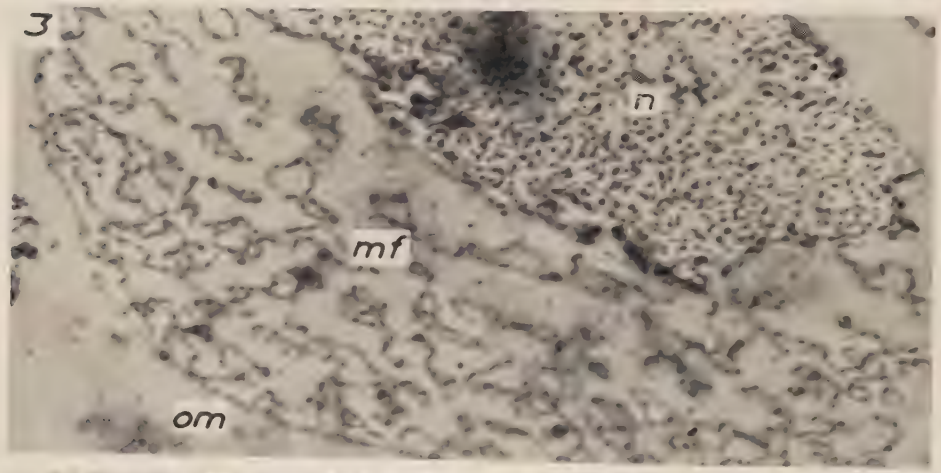
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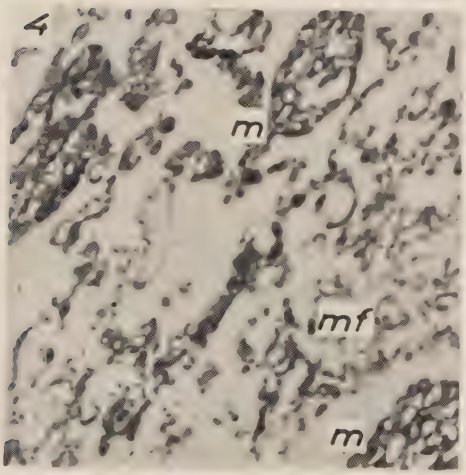
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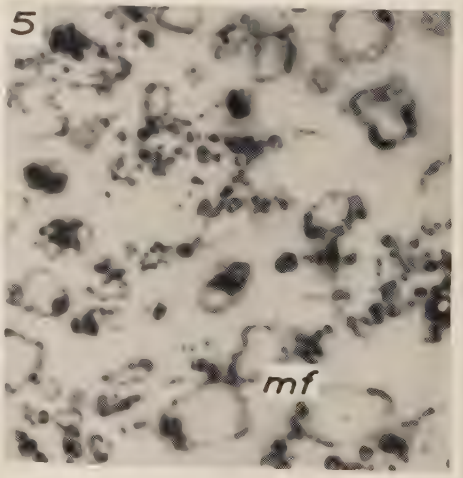
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TABLE I. *ChE activity of subcellular fractions from the electric tissue of* Torpedo.

Fraction	ChE activity ml CO_2/g/hr	ChE activity % of sum	Protein (mg/g)	Specific activity ml CO_2/hr/mg of protein
A (800 × *g*, 15 min)	224.00	60.00	5.60	40.00
B (3000 × *g*, 15 min)	24.80	6.65	1.35	18.40
C (105,000 × *g*, 1 hr)	87.00	23.35	3.39	25.15
D (final supernatant)	37.20	9.90	7.00	4.60
Sum	373.00		17.34	
Whole homogenate	405.00		18.10	22.40
"Purified" fractions				
A^1 (0–600 × *g*)				42.50
B^1 (800–3000 × *g*)				7.72
C^1 (15,000–105,000 × *g*)				39.00
D^1 (final supernatant)				3.86

The "microsomal" fraction, *C*, sedimented at 105,000 × *g* in 1 hour, is mainly formed by small vesicles, accompanied by small dense particles, not well defined (Fig. 5).

The measured diameter of the vesicles found in the different fractions ranges from about 100 to about 400 mμ.

The above-mentioned fractions and the final supernatant were examined in a long series of experiments for their protein and ChE content and were compared with the whole homogenate. Protein was determined by the method of Lowry *et al.* [3], ChE by the manometric method. A typical experiment is illustrated in Table I. In this, as well as in all other experiments, both the highest recovery of ChE and the highest specific activity, on protein basis, were found in fraction *A*. In decreasing

Fig. 1.—Intact electroplaque. Both dorsal (*df*) and ventral face (*vf*) are visible in this field. A well-preserved nucleus (*n*) and some swollen mitochondria (*m*) are present. A system of tubules and vesicles (*ms*) is observed in the cytoplasm close to the dorsal face. Magnification × 8000.

Fig. 2.—Another electroplaque, which shows more developed invaginations on the ventral face (*vf*).

Fig. 3.—First sedimented fraction (*A*). A nucleus (*n*) is partly visible, beside a large fragment of the membrane system (*mf*), which is apparently attached to a piece of the cell outer membrane (*om*). Magnification × 18,000.

Fig. 4.—"Mitochondrial" fraction (*B*). Among some swollen mitochondria (*m*), membrane fragments in form of vesicles (*mf*) are present. Magnification × 18,000.

Fig. 5.—"Microsomal" fraction (*C*). Membrane fragments forming vesicles are the main component (*mf*). Small dense particles, not well defined, are observed besides. Magnification × 18,000.

order, both for recovery and specific activity, followed the "microsomal" fraction *C*, the "mitochondrial" fraction *B* and the final supernatant.

A series of experiments was designed to obtain fractions morphologically as pure as possible. This was achieved by repeated washings and by discarding the intermediate, overlapping fractions, no care being taken for recovery of the activity of the whole homogenate. The specific activity of such "purified" fractions follows the same trend as the previous experiments and emphasizes their results (Table I).

These combined results from morphological and enzymic investigation show that almost all the ChE of the electric tissue is not "soluble", but is associated with particulates from the electroplaque structure. Moreover, a significant correlation is found between the concentration of ChE in the different fractions and the concentration of membrane structures originating from the surface of the electroplaque, mainly from the system of tubules close to the dorsal face.

The finding of a significant concentration of ChE on these membrane structures is of obvious interest because of the suggested role of ChE in the electrogenesis [6, 7].

We wish to express our grateful thanks to Dr. D. Bocciarelli-Steve and to her collaborators at the Department of Physics, Istituto Superiore di Sanità, for the electron microscopical work.

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AMINOPTERIN AND NITROGEN MUSTARD AS MITOTIC INHIBITORS IN MOUSE EAR EPIDERMIS *IN VITRO*

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Received August 23, 1958

It has been reported that both Aminopterin (a folic acid antagonist) and nitrogen mustard (a radiomimetic compound) inhibit cell division in the same manner as colchicine by impairing the spindle and arresting mitosis in metaphase [5, 7, 13]. This conclusion is based primarily upon results obtained on embryonic chick tissues grown *in vitro* [5, 7].

In the rat uterus studied *in vivo* [4], the actions of Aminopterin and nitrogen mustard differ from those of colchicine. Colchicine prevents chromosome movement and acts as a metaphase inhibitor, whereas Aminopterin and nitrogen mustard prevent chromosome duplication and act as preprophase inhibitors [4].

The present study compares the actions of all three compounds on mitosis in mouse ear epidermis cultured *in vitro*. In this system DNA synthesis does not occur [3], and colchicine is used routinely to inhibit epidermal mitosis in metaphase [2]. If Aminopterin and nitrogen mustard are metaphase inhibitors, their action on epidermal mitosis should prove similar to that of colchicine.

Material and Methods.—Mouse ear epidermis was cultured *in vitro* according to the method of Bullough [2]. Adult male mice were used in groups of five, and small pieces of their ears were incubated in a Warburg flask in a saline culture medium in an oxygen gas phase at 38°C. Each flask contained 5 ear fragments obtained from 5 different animals. When glucose was used it was added to the culture medium in a final concentration of 0.02 *M*. When inhibitors were used they were dissolved in saline or distilled water and placed in the side arm of the appropriate Warburg flask. Colchicine dissolved in saline was used in a concentration of 0.016 mg per flask. Aminopterin, 4-amino pteroylglutamic acid (Lederle Laboratories) was dissolved in saline and used in a final concentration of 1.1×10^{-3} *M*. Nitrogen mustard, methyl bis (β chloroethyl) amine hydrochloride (Chicago Toxicity Laboratory) was dissolved in water and used in a final concentration of 10^{-4} *M*.

The ear fragments were incubated for 1 hour to allow all mitoses originally present in the epidermis to pass beyond the metaphase stage. The experimental period began at this point. Inhibitors were tipped into the main vessels of the Warburg flasks, and incubation continued for a further 4 hour period. Mitotic activity was determined at hourly intervals in each experimental group of 5 ear fragments. The numbers of mitoses arrested in metaphase by colchicine were counted in unit lengths of 1 cm in histological sections of epidermis [2]. When colchicine was not used, both metaphase and anaphase figures were included in the mitotic counts. This was done to make sure the effects of Aminopterin or nitrogen mustard were not wearing off [7].

Results and Discussion.—The first 4 experimental conditions listed in Table I demonstrate why colchicine is used routinely to study mitotic activity in mouse ear

TABLE I. *The inhibition of mitosis by colchicine, aminopterin, and nitrogen mustard in mouse ear epidermis cultured at 38°C in an oxygen gas phase.*

Medium	Saline	Saline	Saline + Glucose	Saline + Glucose	Saline + Glucose	Saline + Glucose
Inhibitor	None[a]	Colchicine[b]	None[a]	Colchicine[b]	Aminopterin[a]	Nitrogen mustard[a]
Exptl. Period No. Hrs.						
1	0.0±0.07	0.9±0.2	0.3±0.09	2.2±0.3	0.1±0.06	0.3 ±0.1
2	—	—	0.4±0.1	3.2±0.4	0.3±0.09	0.2 ±0.1
3	—	—	0.7±0.05	6.2±0.7	1.0±0.3	0.04±0.04
4	0.1±0.09	1.2±0.2	1.1±0.3	7.9±0.6	1.4±0.2	0.2 ±0.1

[a] Average No. ±(S.E.) metaphase and anaphase figures per 1 cm unit length.
[b] Average No. ±(S.E.) metaphase figures per 1 cm unit length.

epidermis. The greater number of epidermal cells that enter mitosis in the presence of glucose becomes convincingly apparent when colchicine is introduced. Colchicine arrests mitosis continuously throughout the 4 hour experimental period, both in the presence and absence of glucose.

Unlike colchicine, Aminopterin and nitrogen mustard do not arrest mitosis in metaphase at any time during the experimental period. Epidermal cells enter and complete mitosis, and the results with Aminopterin are comparable to those in which no inhibitor was used. Nitrogen mustard may have acted on prophase [10] and thus prevented cells from entering mitosis during the latter half of the experimental period.

The present study demonstrates that both Aminopterin and nitrogen mustard are dissimilar to colchicine in that they do not inhibit mitosis in metaphase. These results on mouse ear epidermis *in vitro* differ from those obtained by Hughes and Jacobsson in which Aminopterin [5, 7] and nitrogen mustard [5] do inhibit mitosis in metaphase in embryonic chick tissue culture. The explanation that Aminopterin and nitrogen mustard may be acting in different ways in the two different *in vitro* systems presents itself, but does not seem acceptable in view of the biological implications of these compounds [4]. Moreover it even seems unlikely that Aminopterin and nitrogen mustard are acting as chemically defined metaphase inhibitors in chick tissue culture. Test substances are usually applied to actively dividing chick cells grown in a hanging-drop culture and their effects are studied for a short period of 15 minutes [5, 7]. Under such conditions a wide variety of unrelated chemical substances [5], including distilled water [6], inhibit mitosis in metaphase. Dividing chick cells are admittely very sensitive in metaphase [6], and the appearance of mitotic figures following treatment with a chemical compound may therefore be, as Ludford points out, "a clear indication of cell injury: but nothing more" [8].

The situation is quite different in mouse ear epidermis studied *in vitro*. Large numbers of cells are not actively dividing at the time the inhibitor is introduced (Table I).

Epidermal cells gradually enter and complete mitosis with normal frequency [1] during each hour of the experimental period (Table I). Thus the actions of mitotic inhibitors in mouse ear epidermis may be viewed as physiologically significant, especially if they coincide with those observed *in vivo*.

The enormous literature on mitotic inhibitors has been skillfully sorted and cogently used by recent reviewers to dissect and discover the different mechanisms involved in cell division [9, 11, 12, 13]. The present report attempts to contribute to this knowledge by refining the concept of how Aminopterin and nitrogen mustard act to inhibit mitosis.

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